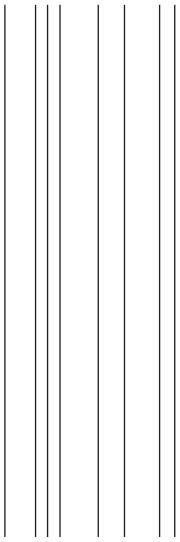

2014 | Faculty of Medicine and Life Sciences

DOCTORAL DISSERTATION

Mitochondrial DNA content in association with the exposome *and its potential as a mediator between exposure and outcome*

Doctoral dissertation submitted to obtain the degree of
Doctor of Biomedical Science, to be defended by

Nicky Pieters

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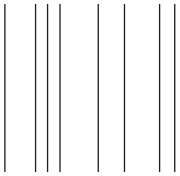

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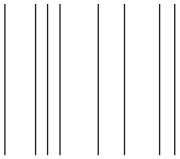

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Nicky Pieters
December 2014

SUMMARY

Introduction and aims

Although universal and unavoidable, ageing does not occur in a uniform way. Ageing is a complex phenotype responsive to both environmental and genetic factors and includes both chronic and acute processes. Among the plethora of biological phenomena affected by ageing, the malfunction and decrease of biogenesis of mitochondria seem to exert some of the most potent effects on the organism. The aims of this thesis focus on mitochondrial DNA content and telomere length as possible intermediates between environmental exposure to ambient air pollution and cardiovascular ageing in the context of the exposome (Chapter 1):

In Chapter 2 and 3, we investigated the association between particulate matter and cardiovascular function in the general population by means of a meta-analysis and also in two cohorts consisting of schoolchildren.

We studied mitochondrial DNA content as a biomarker of biological aging by providing a connection with molecular ageing networks and telomere length (Chapter 4) and with environmental exposures including particulate matter (Chapter 5) and polycyclic aromatic hydrocarbons (Chapter 6).

Finally, we investigated the role of mitochondrial DNA content as a susceptibility marker for particulate matter-induced effects on cardiovascular function in schoolchildren (Chapter 7).

Main results

Chapter 2: Exposure to particulate matter, both at the level of the population and the individual, is associated with acute cardiovascular events such as myocardial infarction. The effect of particulate matter on other cardiovascular parameters, including heart rate variability (HRV) and blood pressure, may be relevant to unravel mechanisms responsible for the adverse effects of particulate matter. The association between HRV and particulate matter (PM) was examined using a meta-analytical approach. Our meta-analysis showed an inverse association between $PM_{2.5}$ exposure and HRV in a population sample of 18 667 persons.

Chapter 3: When performing a literature search on PM-linked cardiovascular effects, we found that most studies focused on adults and elderly. In the context of the 'exposome', we emphasize the importance of studies in children, since many diseases may find their origins early in life.

In a population of schoolchildren, aged 6 to 12 years, an inverse relationship was found between ultrafine particles (UFP) and systolic blood pressure. An interquartile range (IQR) increase in particles with a diameter of 20-30 nm was associated with a 6.35 mmHg increase in systolic blood pressure, compared to a 0.79 mmHg increase in systolic blood pressure for an IQR increase in the total fraction of UFP. These results emphasize the importance of the particle diameter in the effectiveness of particles to cause rapid increases in systolic blood pressure since the effect size decreased with increasing particle diameter.

Chapter 4: The objective of this chapter was to validate mitochondrial DNA content as a marker of biological ageing by providing a molecular link with telomere length and other genes in the telomere-mitochondrial axis of ageing. We found a positive association between telomere length and mitochondrial DNA content. Sirtuin 1 expression, a metabolic sensor involved in mitochondrial biogenesis, was estimated to mediate 40% of the positive association between telomere length and mitochondrial DNA content.

Chapter 5: The molecular markers of ageing identified in chapter 4, including telomere length, mitochondrial DNA content and *sirtuin1* expression, were further evaluated in this chapter by studying the effects of chronic exposure to particulate matter on these biomarkers of ageing. In a study of elderly, we found significant effects of $PM_{2.5}$ exposure on the telomere-mitochondrial axis of ageing. The effects of PM on mitochondrial DNA content were mediated though *sirtuin1* expression.

Chapter 6: Consistent with the results in chapter 5, we found that not only particulate matter but also polycyclic aromatic hydrocarbons (PAH) are able to cause reductions in mitochondrial DNA content. The ability of PAHs to lower mitochondrial DNA content was confirmed *in vitro* in human TK6 cells by exposing the cells to benzo(a)pyrene.

Chapter 7: The role of mtDNA content as a potential underlying factor that modifies susceptibility to cardiovascular effects of PM exposure was shown in schoolchildren where we studied de PM-HRV association. Although we found an inverse association between PM and HRV in the whole study sample, the effect was merely due to children with a low mitochondrial DNA content.

Conclusion

In this doctoral dissertation we showed an overall adverse effect of long term PM air pollution on mitochondrial function in the context of the exposome and we identified mitochondrial function as a susceptibility marker for short-term exposures to PM. Further, we emphasize the importance of children as a susceptible subgroup for the adverse effects of PM, as PM-induced health effects are not limited to persons with underlying disease or elderly but affect the individual from conception onwards.

Further research is necessary to determine the clinical consequences of short-term changes in cardiovascular parameters such as blood pressure and HRV in response to acute elevation in PM concentrations and to determine possible genetic, dietary and lifestyle factors to restore mitochondrial function.

SAMENVATTING

Inleiding en doelstellingen

Hoewel veroudering een algemeen en onvermijdelijk proces is, gebeurt het niet via een vast patroon. De heterogeniteit van het verouderingsproces wijst op de complexiteit en diversiteit ervan en duidt op het gebrek aan adequate biomerkers om de graad ervan te kwantificeren. Tot nog toe is weinig geweten over hoe omgevings- en milieufactoren het moleculaire verouderingsproces beïnvloeden of versnellen.
Mitochondriën en telomeren blijken beiden een cruciale rol te hebben in meerdere verouderingsgerelateerde aandoeningen. Dit proefschrift omvat verschillende epidemiologische studies waarin de rol van mitochondriale DNA inhoud werd onderzocht als biomerker van omgevingsgerelateerde blootstelling (fijn stof) en cardiovasculaire functie in de context van het 'exposoom':

In hoofdstuk 2 en 3 onderzochten we de associatie tussen fijn stof en cardiovasculaire functie in de algemene populatie door middel van een meta-analyse, nadien door het opzetten van twee cohorten bestaande uit schoolkinderen.

We bestudeerden mitochondriale DNA inhoud als biomerker van biologische veroudering door de link te leggen met moleculaire verouderingspathways en telomeerlengte (hoofdstuk 4) en met blootstelling aan fijn stof (hoofdstuk 5) en polycyclische aromatische koolwaterstoffen (hoofdstuk 6).

Tenslotte onderzochten we in hoofdstuk 7 de rol van mitochondriale DNA inhoud als onderliggende factor in de gevoeligheid van fijn stof-geïnduceerde effecten op cardiovasculaire functie in schoolkinderen.

Belangrijkste resultaten

Hoofdstuk 2: Blootstelling aan fijn stof, zowel op populatieniveau als op individueel niveau, is geassocieerd met acute cardiovasculaire events zoals myocard infarct. Het effect van fijn stof op andere cardiovasculaire parameters, waaronder hartritme variabiliteit (HRV) en bloeddruk, kan ook belangrijk zijn om pathofysiologische mechanismen te ontrafelen die aan de basis liggen van de ongewenste effecten van fijn stof op het cardiovasculair stelsel. De associatie HRV-fijn stof werd onderzocht door middel van een meta-analyse waaruit bleek dat HRV invers geassocieerd is met $PM_{2.5}$ blootstelling in een populatiestaal van 18 667 personen.

Hoofdstuk 3: Bij het uitvoeren van de literatuurstudie voor deze meta-analyse werd vastgesteld dat studies omtrent fijn stof en cardiovasculaire effecten zich vooral richten op volwassenen en ouderen. Het 'exposoom' wijst op het belang van studies in kinderen, vermits veel aandoeningen hun oorsprong vinden vroeg in het leven. In een populatie van schoolkinderen, 6 tot 12 jaar oud, werd een inverse relatie aangetoond tussen ultrafijne partikels en systolische bloeddruk. Een IQR stijging in partikels met een diameter van 20-30 nm was geassocieerd met een stijging van 6.35 mmHg in systolische bloeddruk tov een 0.79 mmHg stijging in systolische bloeddruk voor een IQR stijging in de totale UFP fractie. Deze resultaten wijzen op het belang van de partikeldiameter in de effectiviteit van partikels om snelle stijgingen in systolische bloeddruk te veroorzaken. De effectgrootte nam namelijk af met toenemende partikeldiameter.

Hoofdstuk 4: De doelstelling van hoofdstuk 4 was om mitochondriale DNA inhoud te valideren als merker van veroudering door de associatie na te gaan met telomeerlengte en met andere genen in de telomeer-mitochondriale as van veroudering. Er werd een associatie gevonden tussen telomeerlengte en mitochondriale DNA inhoud. Deze associatie werd voor 40% gemedieerd via *sirtuine 1* expressie, een metabole sensor die betrokken is in mitochondriale biogenese.

Hoofdstuk 5: De biomerkers van veroudering geïdentificeerd in hoofdstuk 4, zijnde telomeerlengte, mitochondriale DNA inhoud en *sirtuine 1* expressie,

werden verder geëvalueerd in hoofdstuk 5 door de associatie na te gaan met chronische blootstelling aan fijn stof. In een studie in ouderen werd vastgesteld dat een stijging van de jaarlijkse blootstelling aan $PM_{2.5}$ zorgt voor een reductie van telomeerlengte en mitochondriale DNA inhoud. Verder werd ook aangetoond dat deze reductie in mitochondriale DNA inhoud door $PM_{2.5}$ gemedieerd wordt via *sirtuine 1* expressie.

Hoofdstuk 6: Voortgaand op de resultaten van hoofdstuk 5 werd hier aangetoond dat niet alleen fijn stof maar ook blootstelling aan polycyclische aromatische koolwaterstoffen zorgt voor een reductie van mitochondriale DNA inhoud. Hiervoor werd gebruik gemaakt van een populatie van volwassenen.

Hoofdstuk 7: Terwijl in de vorige hoofdstukken werd aangetoond dat omgevingsblootstellingen een invloed kunnen hebben op mitochondriale DNA inhoud, werd in dit laatste hoofdstuk bepaald of mitochondriale DNA-inhoud een gevoeligheidsmerker of effect-modificator van fijn stof-geïnduceerde gezondheidseffecten is. In een populatie van schoolkinderen werd aangetoond dat mitochondriale DNA inhoud de gevoeligheid voor fijn stof met betrekking tot hartritme variabiliteit beïnvloedt. Wanneer de analyses gestratificeerd werden voor lage en hoge mitochondriale DNA inhoud, kon worden aangetoond dat enkel kinderen met lage mitochondriale inhoud een daling in hartritme variabiliteit vertoonden bij een stijging van blootstelling aan fijn stof.

Conclusie

Dit proefschrift toont een algemene daling van mitochondriale functie na chronische blootstelling aan fijn stof. Verder werd ook aangetoond dat mitochondriale DNA inhoud als een gevoeligheidsmerker kan gebruikt worden na een acute blootstelling aan fijn stof. Tenslotte werd het belang van onderzoek in kinderen aangegeven, vermits zij naast ouderen en personen met onderliggende aandoeningen een gevoelige leeftijdspopulatie vormen voor de effecten van fijn stof.

Verder onderzoek is noodzakelijk om de klinische gevolgen van acute blootstelling aan fijn stof in kinderen te bepalen en om na te gaan welke genetische, voedings- en levensstijlpatronen de mitochondriale functie terug kunnen herstellen.

List of Abbreviations

ACN	Acenaphtylene
ACE	Acenapthene
ANT	Anthracene
AIC	Akaike information criterion
ANS	Autonomic nervous system
BaA	Benzo*(a)*anthracene
BAP	Benzo*(a)*pyrene
BbF	Benzo*(b)*fluoranthene
BkF	Benzo*(k)*fluoranthene
BPE	Benzo*(g,h,i)*perylene
CHR	Chrysene
CI	Confidence interval
COGNAC	COGNition and ultrafine Air pollution in Children
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive proteïn
CV	Coefficient of variation
dBA	Dibenzo*(a,h)*anthracene
EBC	Exhaled breath condensate
ECG	Electrocardiogram
ETS	Environmental tobacco smoke
GSTM	Glutathione S-transferase M
GWAS	Genome-wide association studies
FLE	Fluorene
FLU	Fluoranthene
HEAPS	Health effects of air pollution in Antwerp schools
HDL	High density lipoprotein
HF	High frequency
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HR	Heart rate
HRV	Heart rate variability
IDE	Indirect effect
IL	Interleukin

IQR	Interquartile range
IRC	Interrun calibrator
LSL	Low density lipoprotein
LF	Low frequency
LTL	Leukocyte telomere length
MeSH	Medical subject heading
mtDNA	Mitochondrial DNA
NAP	Napthalene
NDE	Natural direct effect
NFE2L2	Nuclear factor, erythroid 2 like 2
NRF1	Nuclear respiratory factor 1
OXPHOS	Oxidative phosphorylation
NTC	Non template control
PAH	Polycyclic aromatic hydrocarbon
PHE	Phenanthrene
PIY	Indenol*(1,2,3-cd)*pyrene
PM	Particulate matter
$PM_{2.5}$	Particulate matter with an aerodynamic diameter ≤ 2.5 μm
PM_{10}	Particulate matter with an aerodynamic diameter ≤ 10 μm
PM_c	Coarse particles
PPARGC1A	Peroxisome proliferator-activated receptor gamma-coactivator1 alpha
PPARGC1B	Peroxisome proliferator-activated receptor gamma-coactivator1 beta
PYR	Pyrene
qPCR	Quantitative polymerase chain reaction
rMSSD	Square root of the mean squared difference of successive normal to normal intervals
ROS	Reactive oxygen species
RPL0	Acidic ribosomal phosphoproteïn P0
SDNN	Standard deviationof the normal to normal intervals
SES	Socio-economic status

SIRT1	Sirtuin1
SMPS	Scanning mobily particle sizer
TP53	Tumor protein p53
TRF	Telomere repeat binding factor
UFP	Ultrafine particles
YWHAZ	Tyrosine 3-mono-oxygenase activation protein, zeta polypeptide
ZOL	East-Limburg Hospital

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CHAPTER 1

General introduction

1 EXPOSOME

To identify the etiology of chronic diseases it is necessary to determine both the genetic and environmental underlying factors. By using genome-wide association studies (GWAS) the genetic part of chronic diseases has been studied intensively. However, a more complete assessment of environmental exposures is needed to shed light on the intrinsic relationship between the genome and the environment that may lead to chronic diseases. The term 'exposome' refers to the totality of environmental exposures over time from conception onwards.[1, 2] Although full characterization of human exposures is daunting, representative cross-sectional exposure studies could be made to capture critical stages in the human life course.[3] The biomarkers used in the exposome include a wide range of molecules, from xenobiotic and metabolites in blood, to more downstream features at the gene-expression level. Omic's technologies are critical for the characterization of ageing related mechanisms. The term -omics generally refers to the rigorous study of a complete set of biological and non-biological molecules with high-throughput techniques at different levels, including genes (transcriptomics), proteins (proteomics) and metabolites (metabolomics). Omics profiling greatly facilitates the discovery of biomarkers and overlapping patterns in different age groups might unravel common pathways linking early life exposures to age related mechanisms. The non-genetic exposures considered in the exposome can be broadly divided into three categories: internal, specific external and general external. The internal exposome reflects not only chemicals coming from the external environment but those generated endogenously through processes such as inflammation and the ageing process. These internal conditions will have an influence on the cellular environment. The general external environment refers to social determinants of health. Thirdly, the exposome encompasses specific external exposures which include environmental exposures such as air pollution.[3]

2 CHARACTERISTICS OF AIR POLLUTION

Particulate matter (PM) air pollution is an airborne mixture of solid and liquid droplets that vary in number, size, shape, surface area, chemical composition, solubility, and origin.[4] The mixture of air pollutants can be neither

characterized fully nor replicated easily in experiments involving animal or volunteer exposure in the laboratory. These experimental studies tend to investigate toxicological characteristics of single pollutants rather than the complex interactions of the mixture of many pollutants.

PM is generally categorized according to particle diameter. The following PM fractions are commonly recognized, based on aerodynamic diameter: respirable particles, fine particles and ultrafine particles.[5]

2.1 Size distribution

2.1.1 Respirable particles

In the environmental aerosol, most of the total suspended particles belong to the PM_{10} fraction ie particles with an aerodynamic diamer ≤10 μm. These particles are derived primarily from suspension of dust, soil or other crustal materials from roads, volcanoes, farming or mining and they also include sea salts, pollen and molds.[5] Particles with a diameter of ≤10 μm can enter the deeper parts of the respiratory tract.

2.1.2 Fine particles

Fine particles, with a diameter ≤ 2.5 μm, are primarily derived from direct emissions from combustion processes, such as vehicle gasoline and diesel engines, wood burning, coal burning for power generation and industrial processes including smelters and cement plants.[4] Fine particles also contain transformation products, such as sulphate and nitrate, which are generated by conversion from primary sulfur and NOx emissions and secondary organic aerosol from VOC emissions.[5] Due to its physiological and toxicological properties $PM_{2.5}$ is more relevant in affecting human health compared to larger particles. They may be more toxic because they contain sulfates, nitrates, metals and chemicals adsorbed on their surface. Unlike larger particles, $PM_{2.5}$ can be inhaled more deeply into the lungs where they can reach the small airways and alveoli. This fraction will also remain suspended for longer periods of time, and are thus transported over much longer distances and can penetrate indoor environments.

2.1.3 Ultrafine particles

Ultrafine particles (UFP), with a diameter of ≤ 0.1 µm, are typically fresh emissions from combustion related sources such as vehicle exhaust and atmospheric photochemical reactions. Primary UFP have a very short life (minutes to hours) and grow rapidly through coagulation and condensation to form larger particles.[6] UFP are important in morbidity and mortality because of their relatively efficient alveolar deposition and potential to cross the lung-blood barrier. Due to their small size, high number concentration and relatively large surface area per unit mass, UFP carry large amounts of adsorbed or condensed toxic air pollutants which results in a different surface chemistry in comparison with larger particles.[7]

2.2 Polycyclic aromatic hydrocarbons as a component of particulate matter

Polycyclic aromatic hydrocarbons (PAHs) consist of three or more fused benzene rings containing only carbon and hydrogen. These widespread pollutants are formed during incomplete combustion processes. Important sources of PAH exposure are motorized traffic and heating with fossil fuels. Once PAH are emitted to the atmosphere, their molecular weight influences the fate of the gaseous PAH mixture. Heavier PAHs (more than four benzene rings) can absorb to particulate matter, lighter PAHs tend to remain gaseous until removed via precipitation.[8] PAHs present in the environment are not active and are unable to cause carcinogenesis. Only after entering the organism, PAHs can become metabolically active through transformation by cytochrome P450, epoxide hydrolase and dihydrodiol dehydrogenase, which results in the generation of redox-active quinones. These quinones can cause oxidative stress directly.

UFPs have a higher PAH content compared to fine and thoracic particles. The biological potency of UFP is also directly correlated with the PAH content.[9]

2.3 Health effects of particulate matter

A consequence of the complex composition of particulate matter is that it can cause a broad range of health effects. Although the relative risk associated

with current levels of ambient air pollution at the level of the individual is quite small, the overall impact of air pollution on public health is substantial.[10]

The depth of penetration of specific particles is largely dependent on the aerodynamic diameter and may ultimately drive specific health effects. In general, thoracic particles will affect the upper airways, while fine particles reach the smaller airways and bronchioles although they also may deposit in nose and throat. Pollutants can compromise the upper airways by affecting the composition and production of the mucus layer and the function of ciliated cells. Further, particles can affect the sensory cells ending between the epithelia along the airways, affecting the smooth muscle and resulting in hyperreactive airways or increased mucus secretion leading to cough.

In the lower airways, pollutants can affect the alveolar macrophages and the cellular layer forming the lung-blood barrier, a secondary defense line. Oxidative stress is suggested as the main mechanism leading to local and systemic inflammation following inhalation of particles. Local inflammation by particles will affect gas exchange over the lung-blood barrier, chronic inflammation will result in the thickening of the lung-blood barrier. The propagation of the inflammation to the circulation can be due to the transport of inflammatory mediators such as cytokines and interleukins trough the alveolar epithelium into the blood. These inflammatory mediators and autonomic responses can drive a systemic response, which explains the wide range of cardiovascular effects associated with particulate air pollution. Results of animal studies show that UFP can translocate to the circulation, although this is not widely studied in humans.

Since particulate air pollution concentrations can change daily, it is important to make a distinction between acute and chronic effects of exposure. Primarily due to meteorological conditions, air pollution concentrations can change on a short-term basis (hourly), so it is possible to investigate acute effects of air pollution. Chronic effects can be studied due to spatial contrasts rather than temporal changes in air quality.

2.3.1 Short-term effects

Daily changes in particulate matter concentrations will result in a range of cardiovascular and respiratory 'events' including hospital admissions, daily mortality, arrhythmias, myocardial infarction and stroke. Several meta-analyses pooled the effect estimates of mortality of single-city studies. Elevated concentrations of PM_{10} were associated with increased mortality counts.[11] Locations with higher $PM_{2.5}$ concentrations had stronger associations compared to centers with higher PM_{10} concentrations, suggesting that fine particulates are more responsible for the found associations.[4, 11]. Slightly higher effect estimates were found for cardiovascular mortality.[12] Daily variations in disease burden due to particulate pollution is also shown by increases in the number of emergency visits and hospital admissions due to cardiovascular disease, stroke and respiratory disease.[13] At the level of the population, air pollution was recognized as an equally important trigger of myocardial infarction compared to other well accepted triggers such as physical activity, alcohol and coffee consumption.[10] Further, others markers of cardiovascular function, including heart rate variability and blood pressure, also showed differences with acute changes in exposure.[4, 14]

2.3.2 Long-term effects

Several studies support the idea that prolonged $PM_{2.5}$ exposure increases the risk for cardiovascular mortality to an ever greater extent than short-term exposure[14]. Long-term exposure to ambient $PM_{2.5}$ at present-day levels (ie any increase of 10 $\mu g/m^3$) is associated with life expectancy reductions of several months to a few years, probably due to excess cardiovascular mortality.[15] The Harvard Six Cities adult cohort study showed $PM_{2.5}$-mortality associations for all-cause, cardiovascular and lung cancer mortality.[16] In a joint analysis of data from 22 European cohorts within the multicenter European Study of Cohorts for Air pollution Effects (ESCAPE), an association was found between $PM_{2.5}$ and all cause mortality and stroke incidence. PM exposure was also associated with incidence of coronary events.[17-19] In a meta-analysis, considering lung cancer mortality and incidence studies together (since mortality is a valid indicator of incidence), lung cancer incidence was associated with both $PM_{2.5}$ and PM_{10}.[20] PM is considered a Group 1 carcinogen by the IARC. Other health effects that have

been attributed to long-term exposure to PM are chronic respiratory disease incidence and prevalence (including asthma and COPD), chronic changes in spirometric parameters and chronic cardiovascular disease.[21]

2.3.3 Infant/Birth outcomes

Children are more susceptible to the effects of PM exposure compared to adults due to their relatively higher ventilation rate and metabolic turnover, as well as by the fact that some of the organ systems, including the immune system, are still in development.[22] Furthermore, their physical behavior, such as a greater physical activity, spending more time outdoors and their closer proximity to traffic exhaust emission sources compared with adults, might add to their vulnerability towards hypertensive effects of airborne particles.[22] There is abundant evidence that PM exposure has an impact on infants. Studies have found associations on PM with neonatal or infant mortality[23], birth weight[24], prematurity[25], and respiratory endpoints, such as the incidence of asthma or impaired lung development[26]. Also, adverse cognitive development was shown in association with air pollution. Intelligence score were lower in children with higher PAH exposure during pregnancy cancer. In animal studies it was shown that UFP can translocate from nose through the olifactory nerve to the brain, resulting in inflammatory processes resembling degenerative disease, which might propose a mechanism responsible for cognitive effects associated with air pollution.

3 INTERMEDIATE MARKERS OF AGEING

To measure ageing there is a need for adequate biomarkers. A well established marker of biological ageing is telomere length. Recently, mitochondrial DNA (mtDNA) content gained more attention as a possible early marker of ageing.

3.1 Telomere Length

Telomere length is a well-known factor in the development of chronic pathologies and can be considered as a marker of biological ageing. It is determined by chronology as well as by physiology. Human telomeres are complexes of hexameric repeats of DNA at the distal end of the chromosomes

where they provide stability and protection to the coding DNA.[27] Telomere structure is regulated by several proteins including telomerase and the telomeric repeat binding factors 1 and 2 (TRF1 and 2). Telomerase is involved in the replication of telomeric DNA repeats and as such for the maintenance of telomere length. However, telomerase is not active in most mammalian somatic cells, so with each cell division there is an incomplete replication of the DNA-end (end-of-replication problem) where there is a loss of a certain number of telomeric DNA pairs. This natural erosion of telomeres by chronological ageing can be accelerated or delayed by several genetic and environmental factors, and their interaction.

Environmental factors appear to overrate the contribution of the end-of-replication problem through oxidative stress. Oxidative stress is an imbalance between the number of pro-oxidants and anti-oxidants in favor of the former. The primary pro-oxidants are ROS (reactive oxygen species). In normal conditions, ROS are generated as metabolic byproducts of the aerobic mechanism at the level of the mitochondrial electron transfer chain, where superoxide and hydroxyl radicals are produced by the stepwise reduction of oxygen. Under normal conditions the effects of ROS are counteracted by either enzymatic or non-enzymatic anti-oxidants. However, if the effects of ROS are more potent than the anti-oxidative capacity, oxidative DNA damage will occur.[28]

Telomeres have a high guanine content and as such are highly sensible to oxidative stress. The presence of unrepaired nucleotides might interfere with the replication fork and as such increase telomere shortening[29]. Furthermore, telomere DNA is inefficient in the repair of single strand breaks. Therefore telomere shortening and subsequent senescence of cells can be defined as the cumulative burden of oxidative stress.

3.2 Mitochondrial DNA content

Mitochondria are intracellular organelles that are essential for cellular energy provision through the production of adenosine-5´-triphosphate (ATP) via oxidative phosphorylation. They also play a critical role in calcium homeostasis, oxidant signaling, apoptosis, regulation of cell proliferation and metabolism.

Each cell contains approximately 200 to 2,000 mitochondria, each carrying 2-10 copies of mitochondrial DNA (mtDNA) that are bound to protein structures.[30] The human mtDNA is a double stranded, circular molecule of 16.6 kb and contains 37 genes, encoding 13 proteins that are essential for oxidative phosphorylation and ATP production. Intra-mitochondrial synthesis of these proteins requires mtDNA-encoded 12S and 16S rRNAs and 22 tRNAs for their translation.[31]

Not all proteins present in the mitochondria are encoded by the mtDNA. Mitochondria are genetically semi-autonomous because they rely heavily on the tight interplay of nuclear and mitochondrial gene products to maintain mitochondrial functioning. The vast majority of proteins and enzymes that reside in the mitochondria are nuclear gene products. Although mtDNA encodes a small, but essential, subset of the oxidative phosphorylation machinery as well as the RNA components of the mitochondrial translation system required for its expression in the mitochondrial matrix, nuclear genes specify most of the structural catalytic components of the five inner membrane complexes of the oxidative phosphorylation system. Nuclear gene products destined for mitochondria are synthesized on cytosolic ribosomes and imported into the mitochondria by complex multiple mechanisms.[32]

In comparison to the nuclear genome, the mitochondrial genome is more susceptible to damage. The estimated mutation rate of mtDNA is 5-10 times higher compared to nuclear DNA. Partly, the mutation rate depends on oxidative stress generated by intra- and extracellular factors. MtDNA is susceptible to reactive oxygen species (ROS) generated by the respiratory chain due to its proximity. Therefore, mtDNA is particularly vulnerable to the effects of exposure to PM, as oxidative stress was described as an important mechanism by which PM exerts it's adverse effects.[33] The major difference between human nuclear DNA and mtDNA is that the latter lacks protective histones, chromatin structure, and introns. Additionally, the mitochondrial DNA repair mechanisms work less efficiently than that of nuclear DNA. Mutations in the mtDNA occur more frequently in NADH dehydrogenase subunit 4 gene (ND-4) and in the D-loop region. The D-loop is a non-coding area that controls replication and transcription of mtDNA. It contains the site of origin of replication and the place where promotors initiate transcription. Consequently, mutations in this region

can affect the rate of mtDNA replication[34]. On the other hand, defects in mtDNA replication due to mutations in nuclear genes such as the DNA polymerase γ gene, which is the only mtDNA polymerase, are linked with mitochondrial diseases.[32]

Mitochondrial function as exemplified by mtDNA content is associated with different age-related disorders. Blood mtDNA copy number is inversely associated with cognition in elderly women.[35] Further, mtDNA copy number predicts all cause mortality: compared with the first quartile the risk is 17% lower (95% CI: 29 to 2%) in subjects with mtDNA copy numbers above the 25th percentile.[35] MtDNA content is in leukocytes is negatively associated with age. A study by He et al. in two Chinese populatins demonstrated that after stratification by age, mtDNA content was lower in aged subjects (50-59 and 60-70 years) compared with subjects aged 40-49 year.[36] This negative association between age and mitochondrial DNA content was confirmed in a Dutch study.[37]

4 CARDIOVASCULAR FUNCTION

According to the American Heart Association, PM exposure is a primary contributor to cardiovascular morbidity and mortality, often resulting from acute cardiovascular effects after short-term exposure peaks.[14] Heart rate variability (HRV) and blood pressure are both considered to be non-invasive early markers of cardiovascular disease that can be measured from early age on.

4.1 Heart rate variability

HRV is described as fluctuations of the beat-to-beat differences in cardiac rhythm and is thought to be a non-invasive quantitative marker of the autonomic activity controlling heart rate (HR).

The rhythm of the heart is modulated by the sinoatrial node, which is largely influenced by both the sympathetic and parasympathetic branches of the autonomic nervous system (ANS). Parasympathetic influence on heart rate is mediated by the action of the nervus vagus and prevail under resting conditions. However, parasympathetic and sympathetic activity constantly interact to

modulate HR. These changes reflect the heart's ability to respond to physiological and environmental stimuli.

Reduced HRV has been associated with increased risk of myocardial infarction among the population[38] and has been considered as a predictor of increased risk of mortality in patients with heart failure[39]. The relationship between HRV and PM has been investigated to clarify mechanisms underlying the increased risk of cardiovascular disease associated with PM exposure observed in multiple investigations.[40, 41]

4.2 Blood pressure

High blood pressure is a clearly established factor in cardiovascular morbidity and mortality. In contrast to middle-aged and elderly people, relatively little attention has been given to the problem of increased blood pressure in children. Childhood blood pressure is an important predictor of hypertension and cardiovascular disease later in life.[42-46] Although blood pressure is believed to be a complex trait, determined by numerous genetic, biological, lifestyle, social and environmental factors, avoiding or alleviating potentially irreversible adverse factors as early as possible seems reasonable.[47]

In adults, repeated PM-induced elevations in blood pressure also leads to repeated increases in arterial wall stress and may on the long-term result in chronically elevated pressures. Epidemiological evidence for a chronic increase in arterial stiffness in children due to traffic related air pollution, as exemplified by residential traffic related indicators, exists.[48]

5 AIMS

Mitochondrial DNA shows decreases in number and content with advancing age. However, this age-related declines can be overrated by exposure to environmental factors, which can increase the subjects susceptibility to disease.

I hypothesize that mitochondrial DNA content, considered to be a marker of biological ageing, is an important intermediate or modulating factor between

outcomes and environmental exposures. To this end, I collected data in different age groups (children and elderly) and addressed the following objectives:

- The role of mtDNA content in association with environmental exposures
- The role of mtDNA content in association with molecular ageing networks
- The role of mtDNA content as a potential underlying factor that may modify susceptibility to cardiovascular effects of PM exposure, using HRV as an early outcome

We first investigate the link between exposure to ambient air pollution and cardiovascular function in the general population, using a meta-analytic approach (chapter 2). The systematic literature search revealed no studies of heart rate variability in children.

Therefore, we studied the role of blood pressure (chapter 3) and heart rate variability (chapter 7) in children in relation to particulate air pollution. Then, we evaluate mitochondrial DNA content as a marker of biological ageing by providing a molecular link with telomere length (chapter 4) an its association with different environmental exposures (chapter 5 and 6). Finally, we provide evidence for mitochondrial DNA content as an effect-modifier between exposure to particulate air pollution and cardiovascular function, as exemplified by heart rate variability (chapter 7).

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CHAPTER 2

An epidemiological appraisal of the association between heart rate variability and particulate air pollution: a meta-analysis

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Heart 2012

ABSTRACT

Studies on the association between short-term exposure to ambient air pollution and heart rate variability (HRV) suggest that particulate matter (PM) exposure is associated with reductions in measures of HRV but there is heterogenity in the nature and magnitude of these associations between studies. We performed a meta-analysis to determine how consistent this association is. We searched the Pubmed citation database to identify studies on HRV and PM.

Of the epidemiologic studies reviewed, 29 provided sufficient details to be considered. The meta-analysis included 18667 subjects recruited from the population in surveys, studies from patient groups, and from occupationally exposed groups. We computed pooled estimates from a random-effects model.

In the combined studies, an increase of 10 μg/m³ in $PM_{2.5}$ was associated with significant reductions in the frequency-domain measurements, including low frequency (-1.66%, 95%CI:-2.58% to -0.74%) and high frequency (-2.44%, 95% CI:-3.76% to -1.12%) and in time-domain measurements, for SDNN (-0.12%, 95% CI: -0.22% to -0.03%) and for rMSSD (-2.18%, 95% CI:-3.33% to -1.03%). Funnel plots suggested that no publication bias was present and a sensitivity analysis confirmed the robustness of our combined estimates.

Our meta-analysis supports an inverse relationship between HRV, a marker for a worse cardiovascular prognosis, and particulate air pollution.

INTRODUCTION

A recent scientific report from the American Heart Association concluded that particulate matter (PM) is a modifiable risk factor contributing to cardiovascular morbidity and mortality.[1] We provided a novel insight that particulate air pollution is the a relevant trigger for myocardial infarction at the community level.[2-4] Altered cardiac autonomic function as measured by heart rate variability (HRV) is considered to be one of the pathophysiologic pathways through which PM air pollution influences the cardiovascular system.[5, 6] Reduced HRV has been associated with increased risk of myocardial infarction among the population[7] and has been considered as a predictor of increased risk of mortality in patients with heart failure.[8] The importance of this pathway is still under debate.[9, 10] Here we determine whether all the available observational data up to June 2011 support an inverse association and how strong such a relationship between HRV and particulate air pollution may be.

METHODS

Data collection

We followed published guidelines for the reporting of this meta-analysis.[11] A systematic literature search of Pubmed and Web of Knowledge, last accessed on February 15th 2012, was conducted to identify studies of heart rate variability and air pollution published in English. In addition we screened the reference list of all identified relevant publications and review articles found during our literature search. Two search terms were combined using the Boolean operator AND. The first term, air pollution, combined exploded versions of the Medical Subject Headings (MeSH) *air pollution, particulate matter and air pollutants.* The second term was heart rate variability.

Study selection

Two investigators (NP and MP) read all papers and extracted and computerized the relevant information independently (table 1). Reviews, case-reports, pilot studies, animal studies, manuscripts not written in English and studies that reported another association were excluded. Out of 509 initially selected articles, 98 studies reported an association between HRV and air

pollution. If a group published two or more papers based on the same study population (n=31), only that publication providing the most detailed information was included. We selected only studies that used particulate matter with aerodynamic diameter of 10 μm or less (PM_{10}) or 2.5 μm or less ($PM_{2.5}$) as indicators of air pollution. Studies were also excluded when a controlled exposure was used. Where applicable, preference was given to results adjusted for age and heart rate (HR) and additional factors of proven importance.[12] Quality assessment of the selected studies was performed with consideration of the following aspects: study design, response rate, information about responders vs. non responders, sample size, statistical methods, correction for meteorological conditions and personal or local particulate matter assessment (supporting table S1).

Statistical analysis

HRV was evaluated using different time-domain and frequency-domain measures, according to the measurements presented in the included studies. The time domain measures include rMSSD, the square root of the mean squared difference of successive normal to normal intervals, and SDNN, the standard deviation of the normal to normal intervals. Two spectral components were distinguished in the frequency domain measurements: low frequency (LF) power and high frequency (HF) power. Many time and frequency domain variables measured over a 24-hour electrocardiogram (ECG) recordings are strongly correlated with each other.[13] We obtained a meta-analytical effect estimate using random-effects models, because the Cochran's Q statistics showed evidence of heterogeneity greater than expected by the sampling variance alone ($p<0.1$). An effect estimate was derived from the point estimate of each separate study weighted by the inverse of the variance ($1/SE^2$). When we had independent subgroups within a study, each subgroup was treated as a separate study. The effect-size was calculated as a percent change for an exposure increment of 10 μg/m³ in $PM_{2.5}$. The percent change was calculated as $[10^{\hat{}\beta}-1]x100\%$ with 95% confidence intervals (CIs) $[(10^{\hat{}(\beta\pm1.96xSE)})-1]x100\%$, where 10^x is the antilog, β is the estimated regression coefficient, and SE is the standard error of β, when HRV was log-transformed. (1-(Average-β)/Average)x100% was used when there was no transformation. When data for

only PM_{10} were available[6, 14-16], we converted the percent change with the assumption that PM_{10} consists for 70% of $PM_{2.5}$[2, 17] However, other conversion were considered in sensitivity analysis to determine what the effect would be on the overall estimate, supposing PM_{10} consists for 60% or 80% of $PM_{2.5}$.[2] All calculations were independently performed by two researchers [NP, MP].

Sensitivity of the findings was examined by performing the analysis both with and without the occupational studies.[18-20] The analysis was repeated separately for long-term (more than 18 h of analyzable ECG recordings) and short-term (less than one hour of analyzable ECG recordings) ECG recordings. Three studies[20-22] could not be classified according to this distinction and were excluded from the short- and long-term analysis. We determined whether there was a difference in the combined effect size when only studies with a high quality score were included. We also tested the difference between a 24h exposure and 48h exposure on HRV. Further, we evaluated the influence of individual studies on pooled effect sizes by excluding one study at the time. If the point estimate of the combined effect size with one study omitted, lies outside the confidence interval of the overall estimate, the study in question has an excessive influence. We plotted the association size against the standard error of the study. If there is no publication bias, such plot must produce a funnel shape (funnel plot), because the points scatter around the true pooled value with the scattering narrowing as the sample size increases. All p-values are two-sided tests.

A meta-regression was performed to investigate the effect of study design (longitudinal versus cross-sectional), mean age, mean $PM_{2.5}$ concentration, scale of HRV (log versus linear), percentage of men, year of publication and length of analyzed ECG recordings on the combined effect of $PM_{2.5}$ on HRV.

RESULTS

Selection of the studies

Of the studies reviewed 480 reports were excluded. 128 were duplicates, 174 were excluded based on screening the abstract, 24 studies were reviews, 57 studies reported on animal data, one study was a pilot-study, four publications were not written in English, one study were case reports, 20 studies reported another association than PM and HRV, 19 studies with an estimated exposure from other measurements than the particulate air pollution (PM_{10} or $PM_{2.5}$), two did not provide sufficient information to compute the association size, 17 were experimental studies, 31 used the same study population as reports included in the analysis and two studies used PM measurements longer than 24h (supporting figure S1). As a result, we identified 29 studies, comprising 18667 persons, that investigated the association between HRV and particulate matter. This selection includes 25 longitudinal studies [6, 14, 15, 18-40], including one exploratory study [36] and three occupational studies[18-20] and four cross-sectional designs [16, 23, 41, 42]. These are listed in chronological order in Table 1.

Table 1: Characteristics of the studies included in the meta-analysis

Author	Population	#	Age	Men %	Study Design (Measurement pp)	Expo-Sure	Scale of HRV	Length of ECG	PM µg/m³
Liao, 1999[38]	All	26	81 (65-89)[A]	27	Longitudinal (21)	24 h $PM_{2.5}$	Log	6 min	16 ±7[B]
	Without CVD	8	78 (65-84)[A]	37					
	With CVD	18	82 (69-89)[A]	22					
Pope, 1999[14]	CVD/ respiratory disease	7	75 (23-82)[C]	86	Longitudinal (4.1)	24 h PM_{10}	Lin	24 h	84[D]*
Gold, 2000[34]	Elderly	21	73 (53-87)[C]	48	Longitudinal (7.76)	4 h $PM_{2.5}$	Lin	5 min	15 (3-49)[A]
Brauer, 2001[36]	With COPD	16	74 (54-86)[A]	44	Longitudinal (7)	24 h $PM_{2.5}$	Lin	24 h	11 ± 1.[E]
Magari, 2001[18]	Occupational	33	38±13[B]	100	Longitudinal (2)	4 h $PM_{2.5}$	Log	24 h	223 ± 2203[E]
Holguin, 2003[35]	Without hypertension	21	80 (65-96)[A]	52	Longitudinal (18)	24 h $PM_{2.5}$	Log	5 min	30 ± 10[B]
	With hypertension	13	78 (60-88)[A]	30					

Liao, 2004[41]	All Without hypertension With hypertension	4899	62±6[B]	43	Cross-sectional	24 h PM_{10}	HF & LF Log	5 min	17 ± 8[B]*
Pope, 2004[39]	Elderly	88	54-89[F]	43	Longitudinal (2.84)	24 h $PM_{2.5}$	Log	24 h	24 ±20[B]
Riediker, 2004[19]	Occupational young	9	27 (23-30)[A]	100	Longitudinal (4)	9 h $PM_{2.5}$	Lin	10 min	23 (7-39)[A†]
Park, 2004[23]	Elderly(NAS)	497	73±7 [B]	100	Cross-sectional	48 h $PM_{2.5}$	Log	4 min	11 ±8.[B]
Schwartz, 2005 [31]	Elderly	27	74 (64-81)[G]		Longitudinal (12)	24h $PM_{2.5}$	Log	30 min	10 (7-17)[G]
Sullivan, 2005[33]	With CVD Without CVD	21 13	77 (57-85)[C] 78 (72-87)[C]	43 46	Longitudinal (8.38)	24 h $PM_{2.5}$	Ln	20 min	11 (8-16)[G]
Lipsett, 2006[6]	With CAD	19	71.3±6.0[B]	63.2	Longitudinal (12)	6 h PM_{10}	Log	24 h	
Luttmann-Gibson,2006[32]	General	32	71 (54-90)[A]	9	Longitudinal (21.8)	24 h $PM_{2.5}$	Log	30 min	20 (12-25)[H]

Timonen, 2006[30]	With CAD, Amsterdam	37	72±8[B]	65	Longitudinal (10.92)	72 h $PM_{2.5}$	HF Ln SDNN, rMSSD Log	25 min	20 (10-24)[H]
	With CAD,Ertfurt	47	65±8[B]	91					23 (11-27)[H]
	With CAD, Helsinki	47	68±6.[B]	55					13 (8-16)[H]
Vallejo, 2006 [21]	Young	40	27 (21-35)[A]	28	Longitudinal (33)	3.5h $PM_{2.5}$	Ln	13 h	74 (49-111)[G]
Riojas-Rodriuguez, 2006 [22]	General	30	55 (25-76)[A]	83	Longitudinal (111)	11h $PM_{2.5}$	Ln	11 h	47 ± 1.8[E]
Chuang, 2007 [37]	Young	76	21±1.2[B]	67	Longitudinal (3)	24h $PM_{2.5}$	Log	16 min	32 ± 11[B]
Chuang, 2007 [26]	Elderly	46	70±12[B]	41	Longitudinal (168)	4h $PM_{2.5}$	Log	24 h	52 ± 40[B]
Min, 2008 [16]	General	1349	44±22[B]	44	Cross-sectional	24h PM_{10}	Log	5 min	23 ± 13[B]*
Folino, 2009 [25]	With MI	39	60±5[B]	92	Longitudinal (2.7)	24h $PM_{2.5}$	Lin	24 h	34 ± 13[B]
Whitsel, 2009 [15]	Diabetes	770	64 ± 0.1[B]#	0	Longitudinal (3)	24 h PM_{10}	Log	10 sec	20 ±0.1[B]*!
	Impaired fasting glucose	1559							
	Normal fasting glucose	3141							

Zanobetti, 2009 [24]	With CAD	46	57 (43-75)[C]	80	Longitudinal (4)	2 h $PM_{2.5}$	Log	24 h	9 (6-14)[G]
Park, 2010 [42]	General	5465	62±11[B]	47	Cross-sectional	24 h $PM_{2.5}$	Log	30 sec	14 (10-20)[G]
Schneider, 2010 [40]	With CAD	56	66±6[B]	100	Longitudinal (12)	24 h $PM_{2.5}$	Log	24 h	20 ± 15[B]
Suh, 2010 [27]	All	30	65 (55-73)[H]		Longitudinal (7)	24 h $PM_{2.5}$	Log	25 min	
	With COPD	18		40					17 ±9[B]
	With MI	12		83					15 ±8[B]
Wu, 2010 [20]	Occupational	11	36 (27-41)[A]	45	Longitudinal (3)	2 h $PM_{2.5}$	Log	12 h	57 (35-104)[G]
He, 2011 [28]	General	106	56± 8[B]	41	Longitudinal (48)	6 h $PM_{2.5}$	HF,LF Log rMSSD, SDNN Lin	24 h	13.2 ± 14.4[B]
Jia, 2011 [29]	General	30	60 ±5.[B]	40	Longitudinal (116)	24 h $PM_{2.5}$	Log	24 h	38 (0.2-215)[I]

Age, Average PM concentration: A= Arithmetic mean (range), B= Arithmetic mean ± standard deviation, C= Median (range), D= Mean, E= Geometric mean ± Standard deviation, F= Range, G= Median (25%-75% percentile), H= Arithmetic mean (25%-75% percentile), I= Median (1%-99% percentile)

*: Average $PM_{2.5}$ concentration calculated with the assumption that PM_{10} consist for 70% of $PM_{2.5}$, # : Average Age/$PM_{2.5}$ concentration during the first visit, †: Mass, after shift

MI: Myocardial infarction, COPD: chronic obstructive,pulmonary disease, CAD: Coronary artery disease, CVD: Cardiovascular disease

NAS: Normative aging study, MESA: Multi-ethnic study of atherosclerosis

Characteristics of studies

All studies had a time window of PM measurements ranging from 2h up to 1 day before HRV measurements. Length of ECG recordings varied from 10 seconds to 24 hour. Whenever possible, preference was given to long-term ECG recordings (more than 18 h of analyzable ECG recordings). The HRV measurements were expressed on a logarithmic scale in 23 studies[6, 15, 16, 18, 20-24, 26, 27, 29-33, 35, 37-42] and on a linear scale in five studies[14, 19, 25, 34, 36]. One publication used both a linear and logarithmic scale.[28]

Only one study calculated the association between PM-exposure and HRV without reporting possible confounders[36]. One study included potential confounders but did not report which ones.[19] In all but four reports[25, 30, 39, 40] the results were adjusted for age. Eleven publications did not adjust for sex[20, 22, 25, 30, 33, 35, 39]. However in four[15, 18, 23, 40] of these eleven reports, all subjects had the same sex. Most studies also considered additional confounding variables, such as HR[14, 18, 20, 24, 29, 31, 35, 39, 41], BMI[15, 21, 23, 26, 27, 29, 34, 37, 40-42], outdoor temperature[15, 16, 20-34, 37, 39, 41, 42], relative humidity[16, 19-30, 32-34, 37, 39, 41, 42], ethnicity,[15, 24, 28, 32, 34, 41, 42], and (past) smoking[15, 16, 18, 23-25, 40-42].

Summary statistics

We evaluated HRV using both frequency- and time-domain measurements. The combined estimates calculated for a 10 μg/m³ increase in $PM_{2.5}$ showed a decrease of 1.66% (95%CI: -2.58% to -0.74%) and 2.44% (95%CI: -3.76% to -1.12%) for LF and HF, respectively. The corresponding estimates for the time-domain measurements were -0.12% (95% CI: -0.22% to -0.03%) for SDNN and -2.18% (95% CI: -3.33% to -1.03%) in rMSSD (figure 1).

Sensitivity analysis

When the analysis was repeated without three occupational studies[18-20], we found a minor difference in LF, -2.05% (95%CI:-3.21% to -0.88%) and HF, -3.17% (95%CI: -4.92% to -1.41%) for a 10 μg/m³ increase in $PM_{2.5}$ (figure 2). However, the effect of exclusion of these three studies was much larger for

SDNN which results in a combined effect of -1.25% (95%CI: -1.81% to -0.68%).

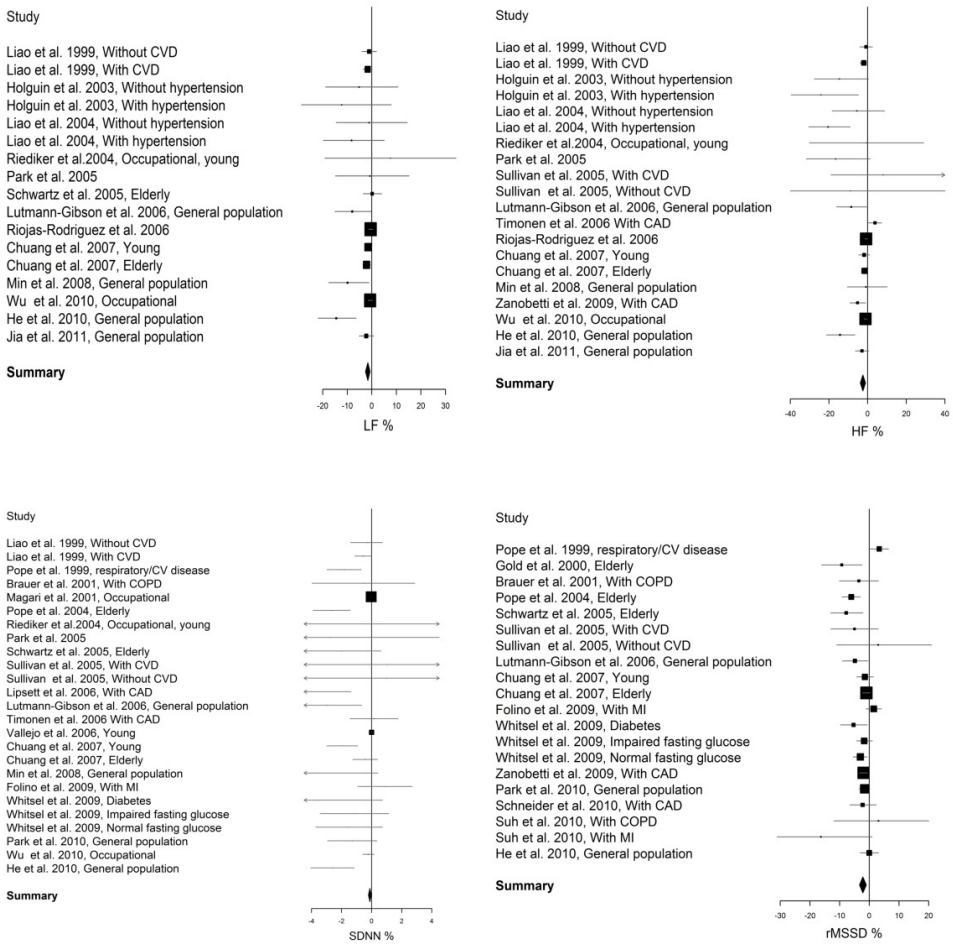

Figure 1: Change in parameter (95% CI) of heart rate variability associated with a 10 µg/m³ increase in $PM_{2.5}$ with inclusion of occupational studies. Squares represent individual groups. The area of each square is proportional to the inverse of the variance.

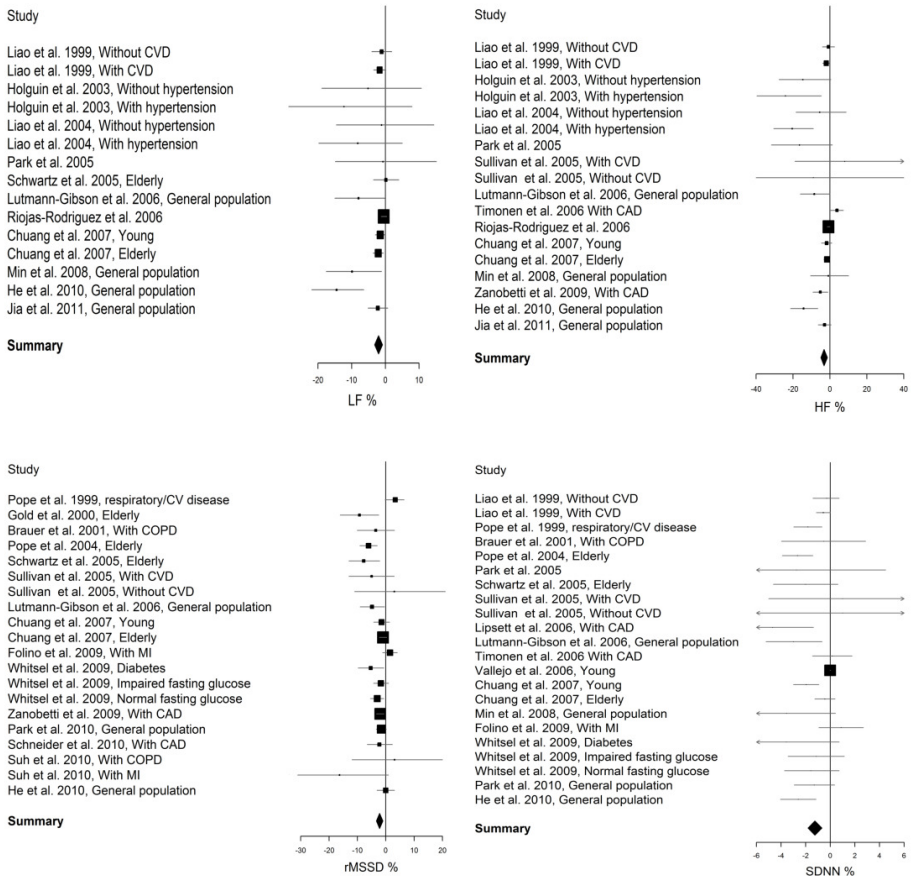

Figure 2: Change in parameter (95% CI) of heart rate variability associated with a 10 µg/m³ increase in $PM_{2.5}$ without inclusion of occupational studies. Squares represent individual groups. The area of each square is proportional to the inverse of the variance.

For LF and HF the majority of studies, 75% en 71% respectively, used short-term ECG recordings. When removing the studies with long-term ECG recordings, [24, 26, 28, 29] we found a decrease of 1.73% (95%CI: -2% to -0.4%) and 1.75% (95%CI: -3.2% to -0.29%) for LF and HF respectively. When repeating the analysis separately for short-term[15, 16, 19, 23, 30-33, 37, 38, 42] and long-

term[6, 14, 18, 25, 26, 28, 36, 39] recordings for SDNN, we observed a decrease in short-term and long-term recordings of -0.73% (95%CI: -1.17% to -0.29%) and -1.39% (95%CI: -2.37% to -0.41%) respectively. When including only short-term[15, 27, 31-34, 37, 42] recordings for rMSSD, we still found a decrease of 3.21 (95%CI: -4.66% to -1.76%) for a 10 $\mu g/m^3$ increase $PM_{2.5}$. However, when only the long-term[14, 24, 25, 28, 36, 39, 40] recordings in seven studies, no statistically significant association was found between rMSSD and $PM_{2.5}$. Excluding specific groups of patients suffering from cardiovascular diseases[6, 14, 24, 25, 27, 30, 33, 38, 40] from the analysis, resulted in a similar combined effect estimate (table 2). The sensitivity analysis was also performed with only studies with a high quality score (more than the median score). The decrease was more pronounced for LF (-2.49%, 95%CI: -4.32% to -0.36%) and HF (-5.74%, 95% CI: -8.82% to -2.66%), similar for rMSSD (-1.89%, 95%CI: -3.08 to -0.71%) but was no longer significant for SDNN (-0.05%, 95% CI: -0.13% to 0.02%). When determining the effect of an 48h exposure, we found a similar decrease in LF, HF and SDNN.

Also, when four studies [16, 23, 41, 42] with a cross-sectional design were excluded, the combined effect estimate was comparable (table 2). When studies with PM_{10}-measurements were excluded from the analysis, the results were similar for all HRV parameters (table 2). The sensitivity of the findings was further examined by removing one study at a time from the analysis and recalculating the combined effect (supporting table S2). For SDNN, we identified two studies[18, 21] that moved the point estimate outside the confidence interval for the overall estimate with all available studies, including the occupational studies . Recalculating the percent change, supposing that PM_{10} consists for 60% or 80% of $PM_{2.5}$, to determine what the effect would be on the overall estimate did not have a significant influence on the overall estimate (data not shown).

Table 2: Sensitivity analysis

HRV		Number of studies included	Number of subjects included	Combined estimate (95% CI)	P value Cochran Q-statistics	I^2 Degree of hetero-geneity (%)
LF	All	14	7172	-1.7 (-2.6 to -0.7)	0.02	45.0
	Excluding occupational studies	12	7152	-2.1 (-3.2 to -0.9)	0.0175	48.8
	Excluding studies with long-term ECG measurements	9	6949	-1.7 (-2.7 to -0.7)	0.4618	0.0
	Excluding groups with patients suffering from cardiovascular disease	16 groups	7154	-1.7 (-2.7 to -0.7)	0.0178	47.7
	Excluding studies with a cross-sectional design	11	427	-1.5 (-2.4 to -0.7)	0.0246	48.7
	Excluding studies with PM_{10} measurements	11	924	-1.5 (-2.4 to -0.6)	0.0373	44.4
	Excluding studies with a low quality score	8	6532	-2.5 (-4.4 to -0.6)	0.0121	57.4
HF	All	16	7356	-2.4 (-3.8 to -1.1)	<0.0001	66.7
	Excluding occupational studies	14	7336	-3.2 (-4.9 to -1.4)	<0.0001	70.1
	Excluding studies with long-term ECG measurements	10	7087	-1.8 (-3.2 to -0.3)	0.0005	62.3
	Excluding groups with patients suffering from cardiovascular disease	16 groups	7140	-2.84 (-4.3 to -1.4)	0.0001	66.1
	Excluding studies with a cross-sectional design	13	611	-1.9 (-3.1 to -0.7)	0.0003	63.4
	Excluding studies with PM_{10} measurements	14	1108	-2.0 (-3.3 to -0.8)	0.0002	63.9
	Excluding studies with a low quality score	9	6585	-5.7 (-8.8 to -2.7)	<0.0001	71.8

SDNN	All	21	13521	-0.1 (-0.2 to -0.03)	<0.0001	74.0
	Excluding occupational studies	18	13468	-1.3 (-1.8 to -0.7)	<0.0001	76.1
	Excluding studies with long-term ECG measurements	11	13167	-0.7 (-1.2 to -0.3)	0.0006	60.7
	Excluding studies with short-term ECG measurements	8	354	-1.4 (-2.4 to -0.4)	<0.0001	84.5
	Excluding groups with patients suffering from cardiovascular disease	19 groups	13286	-0.08 (-0.2 to 0.004)	<0.0001	74.5
	Excluding studies with a cross-sectional design	18	6210	-0.1 (-0.2 to -0.02)	<0.0001	75.6
	Excluding studies with PM_{10} measurements	17	6595	-0.07 (-0.1 to 0.00)	<0.0001	69.2
	Excluding studies with a low quality score	12	12666	-0.05 (-0.1 to 0.02)	<0.0001	73.6
rMSSD	All	16	11437	-2.2 (-3.3 to -1.0)	0.0007	57.8
	Excluding occupational studies					
	Excluding studies with long-term ECG measurements	8	11033	-3.2 (-4.7 to -1.8)	0.1386	31.5
	Excluding studies with short-term ECG measurements	8	404	-1.1 (-2.8 to 0.6)	0.0013	70.4
	Excluding groups with patients suffering from cardiovascular disease	14 groups	11256	-2.8 (-4.0 to -1.5)	0.022	48.3
	Excluding studies with a cross-sectional design	15	5972	-2.3 (-3.6 to -1.0)	0.0004	60.0
	Excluding studies with PM_{10} measurements	15	11349	-1.9 (-3.0 to -0.8)	0.0051	51.5
	Excluding studies with a low quality score	9	11293	-1.9 (-3.1 to -0.7)	0.0643	40.4

HF: High frequency, HRV: Heart rate variability, LF: low frequency, PM_0: particulate matter with aerodynamic diameter of 10 µm or less; rMSSD: square root of the mean squared difference successive normal-to-normal intervals, SDNN: SD of normal to normal intervals

Publication bias

The funnel plots searching for publication bias (supporting figure S2) did not reveal a deficit of small studies with negative results. Meta-regression revealed that study design (longitudinal versus cross-sectional), mean age, mean $PM_{2.5}$ concentration, scale of HRV (log versus linear), percentage of men, year of publication and length of analyzed ECG did not have a significant influence on the combined estimates, with exception for low frequency domain, the effects tended to be less strong in logarithmic studies (p=0.05) and stronger in studies with more men (p=0.04. For HF, the effects tended to be less strong for younger persons (p=0.03).

DISCUSSION

The key finding of the present meta-analysis was that heart rate variability by means of SDNN, rMSSD, HF and LF was decreased after an elevation in exposure to particulate air pollution ($PM_{2.5}$) of 10 $\mu g/m^3$. These effects were calculated using 29 studies that met the inclusion criteria comprising 18667 study participants. We did not observe statistical evidence that the combined effect estimates were largely driven by the three occupational studies.

A reduced HRV is a powerful and independent predictor of adverse prognosis in patients with heart disease[43-45] and in the general population[46, 47]. It is a marker of cardiac autonomic dysfunction and a predictor of sudden cardiac death and arrhythmias.[8] A reduction of HRV has been reported in several cardiological and noncardiological diseases such as myocardial infarction, diabetic neuropathy, cardiac transplantation, myocardial dysfunction, and tetraplegia.[8] HRV measurements are also promising as risk marker for fatigue[48], ageing[49], and stress-situations[50]. Despite the important prognostic power of HRV, it is still not a widely used tool in diagnostic settings, and agreed normative values for HRV remain missing.[51] In terms of clinical significance the association between decreasing HRV with increasing air pollution is still under debate as the decrease in HRV can be a marker of cardiac disease or the cause of increased risk. The underlying mechanisms responsible for the association between fine PM exposure and impaired HRV are not yet fully understood. Substantial

epidemiologic literature links cardiovascular mortality and morbidity to exposures of ambient air pollution.[52, 53] Two main candidate mechanisms are release of prothrombotic and inflammatory cytokines from the lung, and effects on the electrical activity and autonomic function of the heart[54]. HRV is indicative for the physiological responses of the autonomic nervous system, of the combined sympathetic and parasympathetic activity. Alterations in autonomic control of the heart, as represented by HRV, may represent a major pathophysiological mechanism by which air pollution leads to cardiac mortality.[55] Two possible pathways can lead to changes in neural control of the heart affecting HRV by exposure to particulate matter.[55] Inhaled particles can promote a systemic sympathetic stress response that leads to decreased HRV measurements and may cumulate in ventricular tachyarrhythmia. On the other hand, the inhaled particles can stimulate irritant receptors in the lung parenchyma and respiratory airways, which leads to the opposite, an increased parasympathetic vagal response and increased HRV.[55] Our combined estimate showed a decrease in all HRV-parameters, which suggests an overall sympathetic response. The combined effect was most pronounced for HF, which is highly correlated with rMSSD.[14] They both reflect parasympathetic cardial vagal tone. A decrease in SDNN, an overall measure of changes in autonomic tone, also implies an increased risk of cardiac morbidity and mortality.

Felber Dietrich et al. [56] measured the effect of ETS on HRV through 24- ECG recordings in 1218 nonsmokers aged ≥50 years. Individuals who were passively exposed to smoke at home or at work for more than 2 h/day had a decrease of 3.5% in SDNN, 0.14% in HF and 15.35% in LF. Compared with our meta-analytical estimates on HRV and $PM_{2.5,}$ the effects of passive smoking were more pronounced for SDNN and LF, as these effects were -0.12% and -1.66% for a 10 $\mu g/m^3$ increase in $PM_{2.5}$. For HF, the effect for PM was more pronounced, we found an effect of -2.44%. We should be aware that PM exposure is a continuous variable and the expression for a 10 $\mu g/m^3$ increase is a relative small increase, as a difference of 30 $\mu g/m^3$ in acute exposure is still within the range of daily variation in many parts of the world.[2]

Since air particulates can provoke oxidative stress and an inflammatory response in the lung and heart[57], oxidative stress is a potentially important

cellular mechanism. A study by Schwartz et al.[58] concluded that the effects of $PM_{2.5}$ on HF seemed to be mediated by reactive oxygen. The Normative Aging Study has shown a strong effect modification of the PM HRV relationship by obesity and genes that modulate endogenous oxidative stress or xenobiotic metabolism, such as glutathione S-transferase M1, methylenetetrahydrofolate reductase, and the hemochromatosis gene.[58-60] Additional findings suggest protective effects of statins, dietary antioxidants, and B vitamins, as well as omega-3 polyunsaturated fatty acids.[58, 59, 61, 62] These findings imply that pathways that decrease endogenous oxidative stress have a protective effect that alleviates reductions in HRV due to exposure of particulate air pollution. An alternative potential mechanism is provided by Schulz et al.[63] who found that an altered ion-channel function triggered by air pollution in myocardial cells can lead to cardiac malfunction.

Observational studies as included in our meta-analysis do not prove causation. However, repeated observation of an association in different populations and different subgroups showing the same or similar results suggest that the results of a single study are not due to coincidence. Our forest plot showed that the majority of studies showed a decrease in parameters of heart rate variability in association with particulate air pollution. We showed consistent results between different study designs including cross-sectional studies, panel and repeated measure studies, which supports a causal association. Furthermore, we observed heterogeneity between studies but our estimates were robust. Although in general the average concentration of occupational $PM_{2.5}$ exposure was much higher than environmental exposure, exclusion of three occupational studies,[18-20] did not alter the combined estimate.

Although most studies report negative associations between time or frequency domain parameters of HRV and particulate air pollution, the magnitudes of the effect differ among these studies. We addressed the issue of heterogeneity between studies by computing pooled estimates from a random effects model. Differences in magnitude between studies may be due to variation in the composition of PM or length of ECG-recordings but also by including subgroups of populations on anti-inflammatory drugs or lacking anti-inflammatory defence (GSTM null [58]). The effects of $PM_{2.5}$ likely vary depending on pollution sources

and particulate constituents, indeed a multicentre study by Timonen et al.[30] found that the effects of PM on HRV were dependent on local sources of PM. Increases in $PM_{2.5}$ concentration were associated with decreases in HF in Helsinki, but a similar increase in $PM_{2.5}$ was associated with an increase in HF in Ertfurt. Although a 5 minute measurement is recommended and highly reproducible[8, 16], the 24 h measurement includes the nocturnal period during which people in general have a very different autonomic regulation and which is mainly driven by the parasympathetic component[64]. However, when we performed a separate analysis for long-term and short-term recordings, we found a decrease for SDNN and rMSSD in both short- and long-term recordings, although the decrease in long-term recording for rMSSD was statistically not significant (p=0.21). Differences between studies in the HRV particulate air pollution association might also be explained by differences in the disease status of the subjects. Various disease processes (myocardial infarction, diabetes, COPD) as well as physiological conditions, including aging,[65] and drugs (beta-blockers) alter autonomic control, and therefore change HRV. However, when the study groups with subjects suffering from cardiovascular diseases were excluded, the combined estimate did not differ significantly from the effect found when these groups were included. Hence, the association between HRV and PM exposure is not only seen in susceptible subgroups. Contrary, to our meta-analysis, a recent experimental study[9] found no effects of dilute diesel exhaust inhalation for one hour on heart rhythm and heart rate variability in healthy volunteers nor in an 'at-risk' population of patients with stable coronary heart disease. Explanations for the discrepancy between these epidemiological data and negative results in controlled conditions may include too short exposure and difference pollution mixture. Indeed, most of the observational studies included an exposure window of 24 hours. On the other hand, in the observational studies confounding or residual confounding by ambient temperature cannot be excluded. Of the included studies in our meta-analysis 20 (69%) adjusted for meteorological conditions.

To determine whether the combined effect is influenced by a particular publication, a sensitivity analysis was performed. HRV-parameters were not strongly determined by one study. When the most influential study was excluded

from the analysis, statistical significance did not appear but the combined estimate was on average 0.30% higher for the HRV components studied.

Our main analysis included only studies which used a lag of ≤ 24h of $PM_{2.5}$ or PM_{10} exposure. However, we conducted a separate meta-analysis with studies reporting the effect of a 48h exposure[14, 16, 23, 30, 37]. The combined effect estimate for 48h was similar to the effect found for 24h when occupational studies were excluded for LF, HF and SDNN. The effect of a 48h exposure could not be calculated for rMSSD due to the lack of studies reporting rMSSD.

The present results should be interpreted within the context of their limitations. First, the analysis was not adjusted for variation in length of ECG-recordings. On the other hand a sensitivity analysis with the different lengths of the ECGs did not reveal differences in combined effect-sizes according to length confirmed the results for LF, HF and SDNN. For rMSSD, the analysis stratified for short- and long-term recordings showed only significant results for short-term recordings. Third, if there was heterogeneity in the reporting strategy between studies, we had to address this issue by calculating the beta-coefficients to percentages and calculated $PM_{2.5}$ from PM_{10} by using the formulas as given in the methods section. Also, different study designs were used in the combined estimate. Nevertheless, in the sensitivity analysis, we confirmed the robustness of the overall estimate by including only panel studies. In other words the overall estimate was not strongly influenced by the included studies with a cross-sectional nature. And last, generally accountable for meta-analysis is a publication bias in that studies with a positive result are more likely to be published than negative results . The funnel plots searching for publication bias did not reveal a deficit of small studies with negative results, suggesting that overall there was no publication bias.

The present meta-analysis shows an overall statistically significant inverse association between parameters of heart rate variability and exposure to particulate air pollution, which might be relevant in biological terms. Indeed, the putative mechanisms of the acute effects of PM include sympathetic activation/parasympathetic withdrawal leading to hemostatic and hemodynamic changes that are recognized to increase the risk of cardiovascular events.

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SUPPORTING INFORMATION

Table S1: Quality assessment of the selected studies

Reference	Participant characteristics	Response higher than 75%	Information about responders vs. non responders	Sample size higher than 400 persons	Repeated measures	Statistical methods
Liao et al. 1999 [12]	1	0	0	0	1	1
Pope et al. 1999 [13]	1	NA	NA	0	1	1
Gold et al. 2000 [14]	1	0	0	0	1	1
Brauer et al. 2001 [15]	1	0	0	0	1	1
Magari et al.2001 [16]	1	UD	0	0	1	1
Holguin et al. 2003 [17]	1	1	0	0	1	1
Liao et al. 2004 [18]	1	0	0	1	0	1
Pope et al. 2004 [19]	1	UD	0	0	1	1
Riediker et al. 2004 [20]	1	UD	0	0	1	1
Park et al. 2004 [21]	1	NA	NA	1	0	1
Schwartz et al. 2005 [22]	1	UD	0	0	1	1
Sullivan et al. 2005 [23]	1	UD	0	0	1	1
Lipsett et al. 2006 [6]	1	UD	0	0	1	1
Luttmann-Gibson et al.2006 [24]	1	0	0	0	1	1
Timonen et al. 2006 [25]	1	NA	NA	0	1	1
Vallejo et al. 2006 [26]	1	1	1	0	1	1
Riojas-Rodriguez et al.2006 [27]	1	UD	0	0	1	1
Chuang et al.2007 young [28]	1	1	0	0	1	1

Chuang et al. 2007 elderly [29]	1	UD	0	0	1	1
Min et al.2008 [30]	1	UD	0	1	1	1
Folino et al.2009 [31]	1	1	0	0	1	1
Whitsel et al.2009 [32]	1	UD	0	1	1	1
Zanobetti et al. 2009 [33]	1	UD	0	0	1	1
Park et al. 2010 [34]	1	1	0	1	0	1
Schneider et al. 2010 [35]	1	UD	0	0	1	1
Suh et al. 2010 [36]	1	UD	0	0	1	1
Wu et al. 2010 [37]	1	UD	0	0	1	1
He et al. 2011 [38]	1	1	UD	0	1	1
Jia et al. 2011 [39]	1	UD	0	0	1	1

Reference	**Personal particulate matter assessment**	**Local particulate matter assessment**	**Correction for meteorological variables (temperature, relative humidity)**	**Correction for individual variables (Age, heart rate)**	**Overall quality score**
Liao et al. 1999 [12]	0	1	0	0	4
Pope et al. 1999 [13]	0	0	0	0	3
Gold et al. 2000 [14]	0	0	1	0	4
Brauer et al. 2001 [15]	0	0	0	0	3
Magari et al. 2001 [16]	1	0	0	1	5
Holguin et al. 2003 [17]	0	1	0	1	6

Liao et al. 2004 [18]	0	0	1	1	5
Pope et al. 2004 [19]	0	0	1	0	4
Riediker et al. 2004 [20]	0	1	0	0	4
Park et al. 2004 [21]	0	0	1	0	4
Schwartz et al.2005 [22]	0	0	1	1	5
Sullivan et al. 2005 [23]	0	1	1	0	5
Lipsett et al.2006 [6]	0	0	0	0	3
Luttmann-Gibson et al.2006 [24]	0	0	1	0	4
Timonen et al. 2006 [25]	0	0	1	0	4
Vallejo et al. 2006 [26]	1	0	1	0	7
Riojas-Rodriguez et al. 2006 [27]	1	0	0	0	4
Chuang et al. 2007 young [28]	0	1	1	0	6
Chuang et al. 2007 elderly [29]	0	0	1	0	4
Min et al. 2008 [30]	0	0	1	0	5
Folino et al. 2009 [31]	1	0	1	0	6
Whitsel et al. 2009 [32]	0	0	1	0	5
Zanobetti et al. 2009 [33]	0	0	1	1	5
Park et al. 2010 [34]	0	0	1	0	5
Schneider et al. 2010 [35]	0	0	0	0	3
Suh et al. 2010 [36]	1	0	1	0	5
Wu et al. 2010 [37]	1	0	1	1	6
He et al. 2011 [38]	1	0	1	0	6
Jia et al. 2011 [39]	0	1	1	1	6

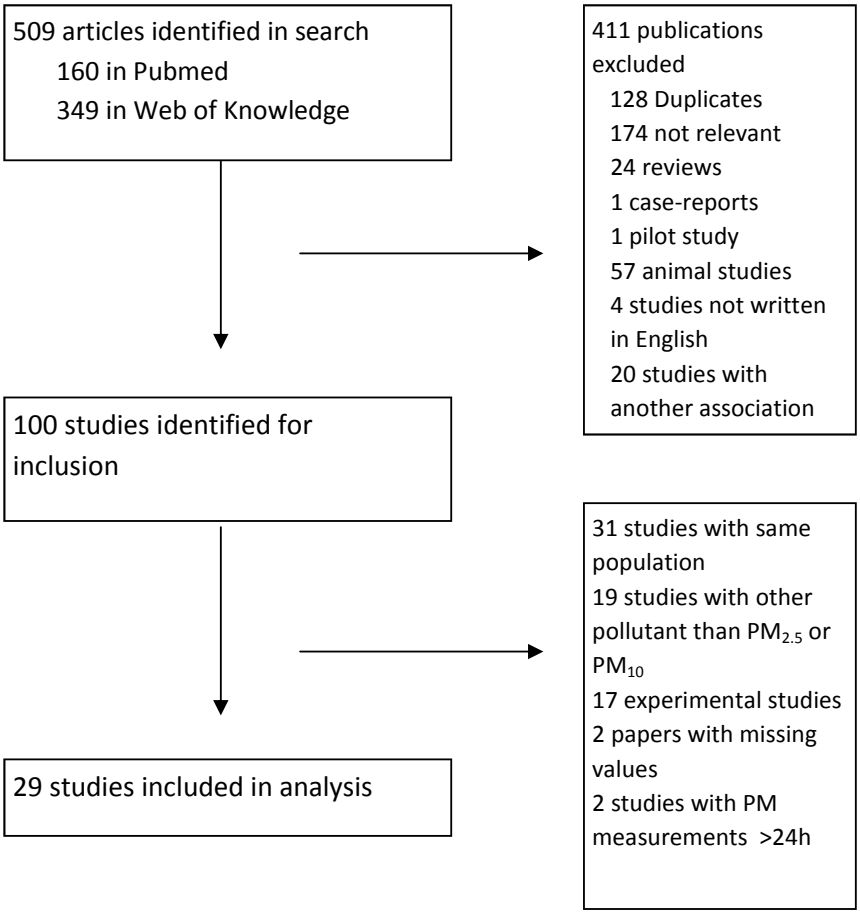

Figure S1: Flow chart of included studies

Table S2: : Change in heart rate variability (95% CI) associated with a 10 µg/m^3 increase in $PM_{2.5}$ after removing the most influential studies

HRV parameter	Inclusion of occupational studies	Largest combined effect estimate in % when excluding one study [Ref]	Smallest combined effect estimate in % when excluding one study [Ref]
LF	Yes	-2.01 %(95%CI: -3.11% to -90%)[20]	-1.23% (95%CI: -1.85% to -0.61%)[28]
	No	-2.42% (95%CI: -3.72% to –1.13%) [37]	-1.51% (95%CI:-2.32% to -0.40%) [[28]
HF	Yes	-3.14% (95%CI:-4.84% to -1.43%) [20]	-1.96% (95%CI:-3.16% to -0.76%) [28]
	No	-4.05% (95%CI: -6.29% to -1.81%) [38]	-2.49% (95%CI: -4.10% to -0.87%) [41]
SDNN	Yes	-1.09% (95%CI:-1.54% to -0.63%) [21]	-0.1%(95%CI:-0.19% to -0.02%) [39]
	No	-1.36% (95%CI:-1.94% to -0.78%) [25]	-1.11% (95%CI:-1.66% to -0.57%) [39]
rMSSD	/	-2.43% (95%CI:-3.5% to -1.36%) [14]	-1.86% (95%CI:-2.96% to -0.76%) [39]

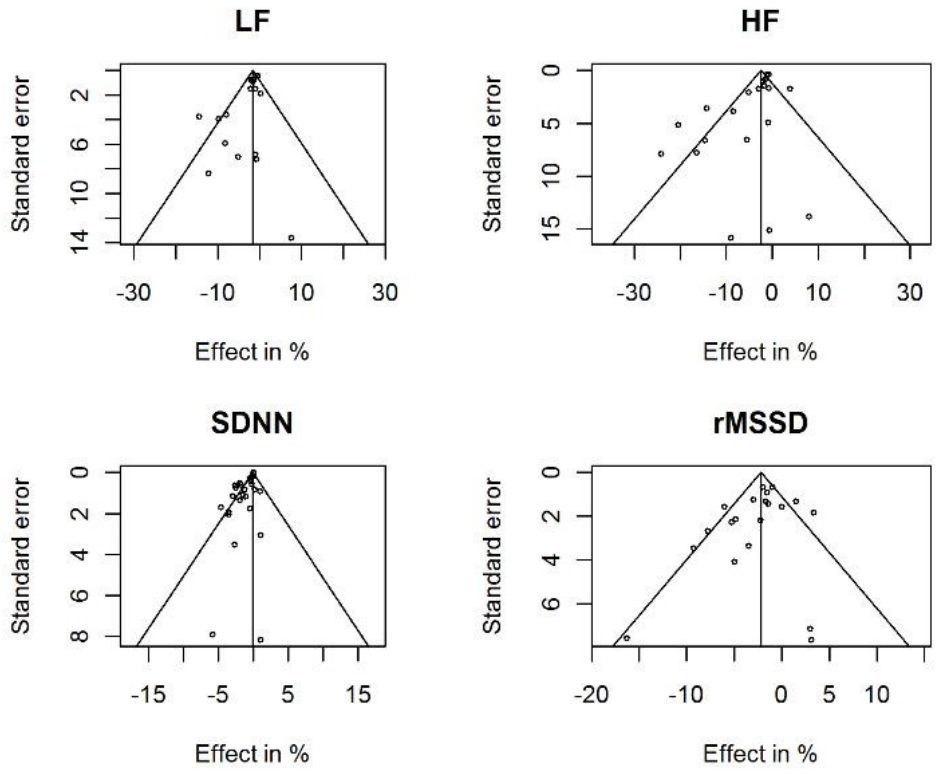

Figure S2: Funnel plot

CHAPTER 3

Acute responses of children's blood pressure to particulate air pollution exposure at school: from nano-sized to coarse particulates

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ABSTRACT

Ultrafine particles (UFP) may contribute to the cardiovascular effects of particulate air pollution, partly because of their relatively efficient alveolar deposition. We assessed associations between blood pressure and short-term exposure to air pollution in a population of school children.

In 130 children (aged 6-12 years) blood pressure was determined during two periods (spring and fall 2011). We used mixed models to study the association between blood pressure and ambient concentrations of particulate matter and ultrafine particles measured in the schools' playground. Independent of gender, age, height and weight of the child, parental education, neighborhood socio-economic status, fish consumption, heart rate, school, day of the week, season, wind speed, relative humidity and temperature on the morning of examination, an interquartile range (860 particles/cm³) increase in nano UFP fraction (20-30 nm) was associated with a 6.35 mmHg (95% CI: 1.56 to 11.14; p=0.01) increase in systolic blood pressure. The corresponding effect size for the total UFP fraction was 0.79 mmHg (95%CI: 0.07 to 1.51; p=0.03) while no effects on systolic blood pressure were found for the nano-sized fractions with a diameter larger than 100 nm, nor $PM_{2.5}$, PM_{coarse} and PM_{10}. Diastolic blood pressure was not associated with any of the studied particulate mass fractions.

Children attending school on days with higher UFP concentrations (diameter smaller than 100 nm) had higher systolic blood pressure. This rapid or acute effect is largely dependent on particle size and was independent of the $PM_{2.5}$ mass concentration.

INTRODUCTION

Air pollution is a complex mixture of solid particles and gases. Different size distribution modes can be identified for airborne particles. Coarse mode particles, with diameters larger than 2.5 µm, are generally produced by mechanical processes. The formation of ultrafine particles (UFP, particles with a diameter less than 100 nm) is often related to combustion or gas-to-particulate conversion. In contrast to $PM_{2.5}$ (particles with a diameter less than 2.5 µm), long-range transport is usually not a major source of UFP in urban areas because of the short lifetime of UFP.[1] Relative to larger particles, UFP demonstrate greater cytotoxicity and inflammatory capacity per mass basis. UFP produce a significant inflammatory response in lung cells. The pulmonary inflammatory response induced by UFP may trigger or enhance systemic effects including those of the cardiovascular system.[2, 3]

Short-term elevation in particulate air pollution is associated with an increased risk for acute myocardial infarction and stroke.[4] One important biological mechanism contributing to these short-term associations is an air pollution-mediated pro-hypertensive response.[5] Epidemiological studies demonstrate that present-day levels of PM are capable of elevating blood pressure in elderly.[6] A French study in pregnant women showed that a interquartile range increase in PM_{10} was associated with a 1.1% increase in diastolic blood pressure during the first trimester of pregnancy.[7] Human exposure studies show that blood pressure increases within only hours of exposure and that blood pressure can remain high until 24h post-exposure.[8] Chuang et al. found increases in systolic and diastolic blood pressure in association with 1-3h moving averages of submicrometer particles with a size range of 0.02 to 1 µm in patients with lung function impairment.[9] An interquartile range increase of 20.8 µg/m³ in 24h mean outdoor $PM_{2.5}$ was associated with an increase in pulse pressure of 4.0 mmHg in elderly taking anti-hypertensive medication.[6] In a population of adults, present day levels of PM_{10} and nitrogen dioxide were associated with an increase in blood pressure.[10] So far, no acute changes in blood pressure in association with PM_{10}, $PM_{2.5}$, nitrogen dioxide and ozone have been found in school children.[11-13]

Studies of the effects of air pollution in children have mainly investigated neonatal or infant mortality[14], birth weight[15], prematurity[16] and respiratory endpoints, such as the incidence of asthma or impaired lung development[17]. Limited research has been done to evaluate cardiovascular parameters, including peripheral blood pressure, in relation to acute changes in urban pollution in children. In this study we investigate changes in blood pressure of children in association with their exposure at school to a broad span of particles ranging from the nano to the coarse size. Furthermore, to investigate possible underlying mechanisms, inflammatory markers were measured in exhaled breath condensate (EBC), a relevant matrix to study immediate or short-term responses to air pollution.

METHODS

Study population

Children aged 6-12 years were recruited at two primary schools in Antwerp, Belgium, within the framework of the HEAPS (Health effects of air pollution in Antwerp schools) study.[18] Children were eligible for inclusion if 1) they were living for at least one year at their current address, 2) they were not planning on moving during the next year, 3) there was no indoor smoking in their houses and 4) their parents were able to complete a questionnaire in Dutch. A subset of 130 children was selected to participate in a study measuring blood pressure. Written informed consent was requested from the parents. Each child was examined twice in periods of ca. 26 weeks apart: the first sampling period fell within the spring season 2011 (May 10th- June 19th) whereas the second period fell within fall 2011 (November 3th- December 13th). Six children no longer participated in the second campaign due to change of school or parental refusal. On three study days no data on UFP were obtained. This resulted in repeated measurements for 90 children and single measurements for 40 children. Additional information such as the child's address, age, parental educational status, fish consumption and travel time from home to school was obtained via questionnaires filled out by the parents. The individual socio-economic status (SES) was defined as the highest level of education of the mother or the father and was categorized as low (high school not finished), middle (high school finished) and high (higher education or university). In

addition, neighborhood SES was assessed using median household income of for the year 2011, provided at the statistical sector level by the Belgian National Institute for Statistics. Fish consumption was coded as a categorical variable: never/rarely, one or two times/week and three or more times/week. Since only a small number of children (4%) had a fish consumption of 3 or more times/week, this category was taken together with one or two times/week. Data on travel time were missing for nine children. The study was approved by the Medical Ethics Committee of Antwerp University.

Clinical measurements

The sampling was organized on weekdays between 09.00 AM and 01.00 PM in the school. Height and weight were measured, while children were not wearing shoes but were fully clothed. Underweight, normal weight, overweight and obesity were determined based on 'Vlaamse groeicurven 2004'[19], which take age and gender into account. After the children had rested for five minutes in a sitting position, a study nurse measured blood pressure by making five to seven consecutive readings using an automated blood pressure instrument (Stabilo Graph, Germany) with a pediatric cuff. The guidelines of the European Society of Hypertension were followed for the measurement of blood pressure. [20] The first blood pressure measurement was excluded. The mean of the remaining blood pressure measurements was used for analysis.

Markers of inflammation

Interleukin (IL) 1β, IL-6, IL-8 and IL-10 were measured in EBC. EBC was collected using an RTube™ sampling device (Respiratory Research, Inc., Austin, TX, USA). The RTube™ was mounted with an aluminum sleeve that was cooled on dry ice for at least 10 minutes before collection. Subjects were asked to breath tidally through a mouthpiece connected to the tube during 15 minutes, yielding approximately 1 mL of EBC. No food was taken 1 h prior to collection. After collection the EBC was immediately divided in aliquots of 250 µL, using 1.5 mL protein LoBind tubes (Eppendorf, Hamburg, Germany). Samples were kept on dry ice and stored at -80 °C until further analysis. IL-1β was analyzed using a Meso Scale Discovery Ultra-Sensitive Kit (Meso Scale Discovery, Rockville, MD, USA) which had a detection limit of 50 fg/mL. Samples below the detection limit

(21% of all samples) were given a value of 25 fg/mL. Plates were read using a SECTOR® Imager 6000 instrument (Meso Scale Discovery).

Air pollution measurements

Ambient concentrations of UFP, $PM_{2.5}$ and PM_{10} were measured in the playground of both schools during the study period; data from 08.00h to 10.00h AM were used to assess acute effects. Air pollution monitoring devices were placed on ground level of the playground. Air was sampled at a height between 1.5 and 2.5 m.

UFP was measured with a Scanning Mobility Sizer (SMPS, 3080, TSI, USA) and UFP monitor (3031,TSI, USA). This latter device allows to determine the number of particles per size fraction (20-30 nm, 30-50 nm, 50-70 nm, 70-100 nm, 100-200 nm and >200 nm). Total UFP fraction was defined as the sum of all separate fractions. The SMPS has a higher size resolution of 64 size bins per decade. Both instruments were compared and the SMPS size distribution was recalculated to the UFP 3031 size bins.

$PM_{2.5}$ and PM_{10} concentrations were measured with an optical counter (1.108, Grimm, USA), with a sensitivity of 1 particle count/L, a mass resolution of 0.1 µg/L and a reproducibility of 2%. This device has 15 different size channels for particles with a size between 0.3 and 20 µm. During the monitoring campaign the data were validated to the reference (filter) method using a low volume sampler (Partisol 2025, Thermo Scientific, USA), equipped with a $PM_{2.5}$ sampling inlet at a flow rate of 16.7L/min. PM_{coarse} was defined as the PM_{10} fraction minus the $PM_{2.5}$ fraction.[21]

UFP data in the playground of their own school were missing for 30 children in the first sampling period and 26 children in the second sampling period. These missing values were imputed using UFP measurements at the playground of the other school (at a distance of 2800 m). Comparison of simultaneous measurements on both locations showed no significant difference. Temperature, relative humidity and wind speed from 08.00h to 10.00h AM were obtained from a local fixed and validated measuring station (42R801) located 2 and 3 km from the examination locations.

Residential distance to major roads

The children's home addresses were geocoded by address; the accuracy was visually inspected using Google Maps and coordinates were manually adapted when they differed from the actual position of the residence. Residential distance to major roads (based on road classification, not intensity) was calculated in ArcGIS 9.3 using the Tele Atlas MultiNet.

Statistical analysis

Statistical analyses were conducted using the SAS statistical package, version 9.3 (SAS Institute, Cary, NC, USA) and GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA). Mann-Whitney U and Fisher exact tests were used to assess differences between the two schools for continuous and categorical data respectively. The association between blood pressure and air pollution was examined by treating the pollutants as continuous variables. We used mixed models with random subject effects accounting for repeated measures, assuming a compound symmetry covariance structure. Models were adjusted for the following fixed effects: gender, age, height and weight of the child, parental education, neighborhood socio-economic status, fish consumption, heart rate, school, day of the week, season, wind speed, relative humidity and temperature on the morning of examination. Time-invariant subject characteristics (such as gender and parental education) were included to permit the assumption of a normally distributed random subject intercept. The shape of the association between blood pressure and air pollution and temperature was explored by using natural cubic splines with different numbers of degrees of freedom. Model fit was assessed by using the Akaike Information Criterion (AIC). The interaction term between season and temperature was explored. For the UFP variables, the single-pollutant analyses described above were repeated with additional adjustment for $PM_{2.5}$ concentrations in the model.

In a series of sensitivity analysis, the model was additionally adjusted for travel time from home to school and for residential distance to major roads. Further the analyses were repeated excluding imputed UFP data, excluding days with low UFP concentration (total UFP <5000 particles/cm^3) and excluding days with high UFP concentrations (total UFP >10000 particles/cm^3).

Associations between IL-1β, a marker of inflammation, and air pollution were examined by mixed models adjusted for the same confounders as before except heart rate. Estimates (with 95% confidence intervals) are presented for interquartile range (IQR) increases in pollutant concentrations.

RESULTS

Descriptive characteristics of the study population are given in Table 1, separately for the two schools. Overall, the study population consisted of 130 children aged 6-12 years (50% girls). Mean (SD) height was 135.8 (10.1) cm and mean (SD) weight amounted 30.9 (7.9) kg (Table 1). Systolic and diastolic blood pressure averaged (SD) 107.1 (8.8) and 60.8 (7.1) mmHg respectively. The mean accumulated concentration for UFP, $PM_{2.5}$, PM_{coarse} and PM_{10} fractions, measured on the different examination days between 8.00h and 10.00h AM and the corresponding temperature from 08.00h to 10.00h AM are given in figure 1 and 2. The mean relative humidity was 70.3% and 84.0% for the first and second sampling period, respectively (supporting figure S1). The distribution for the different UFP fractions and PM is given in Table 2. Correlations between the different size fractions of UFP and the coarse size fractions are shown in Supporting Table S1. The largest UFP fractions (100-200 nm and >200 nm) were correlated with $PM_{2.5}$ and PM_{10}, in contrast the smallest UFP fractions (20-30 nm) was not correlated with $PM_{2.5}$ or PM_{10}.

Comparing the fit of the models with a different number of degrees of freedom for air pollution, the association between blood pressure and air pollution showed linearity. Temperature, however, was inversely associated with blood pressure at temperatures above approximately 12°C and no association at lower temperatures. Models with an interaction term between temperature and season (corresponding to a piecewise linear model with a breakpoint at 12°C) provided the best model fit and were used in further analyses.

Table 1: Characteristics of the study population

Characteristics	**School 1**	**School 2**
Number of subjects	63	67
Girls	31 (49%)	34 (51%)
Age, years	9.0 ± 1.5	8.9 ± 1.4
Height, cm	135.8 ± 10.7	135.8 ± 9.6
Weight, kg	31.8 ± 8.8	30.1 ± 7.0
BMI, kg/m^3		
Underweight	7 (11%)	11 (6%)
Normal weight	45 (72%)	52 (78%)
Overweight	9 (14%)	2 (3%)
Obese	2 (3%)	2 (3%)
SES indicators		
Parental education (individual level)		
Low	2 (3%)	2 (3%)
Middle	15 (24%)	13 (19%)
High	46 (73%)	52 (78%)
Income households (aggregated statistical sector, in euro)	20 407.3	22 195.9*
Fish consumption		
Never/rarely	16 (25%)	11 (16%)
1 or more times a week	47 (75%)	56 (84%)
Travel time from home to school, min	8.9 ± 4.3	13.3 ± 9.2*

Systolic blood pressure, mmHg	110.4 ± 8.2	104 ± 8.1*
Diastolic blood pressure, mmHg	63.1 ± 6.7	58.6 ± 6.8*
Heart rate, beats/min	83.6 ± 10.1	83.8 ± 10.7

Data are given as mean ± SD or number (%).*Significant difference between the two schools

Table 2: Exposure characteristics.

PM Fraction	**Min**	**25th P**	**75th P**	**Max**
20-30 nm, #/cm^3	582	1018	1878	2084
30-50 nm, #/cm^3	603	1637	2349	4116
50-70 nm, #/cm^3	358	947	1486	2886
70-100 nm, #/cm^3	203	673	1031	3035
100-200 nm, #/cm^3	240	666	908	4601
>200 nm, #/cm^3	33	143	279	1205
Total UFP, #/cm^3	2020	5538	7204	17701
$PM_{2.5}$, µg/m^3	2	8	43	53
PM_c, µg/m^3	1	5	14	34
PM_{10}, µg/m^3	5	21	45	64

UFP: ultrafine particles, PM: particulate matter

In spring (temperatures above 12°C), the estimated decrease in blood pressure for a 1°C increase in temperature is -1.35 mmHg (95% CI: -2.08 to -0.63, p=0.0003), whereas in autumn (temperatures below 12°C) the effect of temperature was not significant (-0.09 mmHg; 95% CI: -0.58 to 0.40, p=0.72).

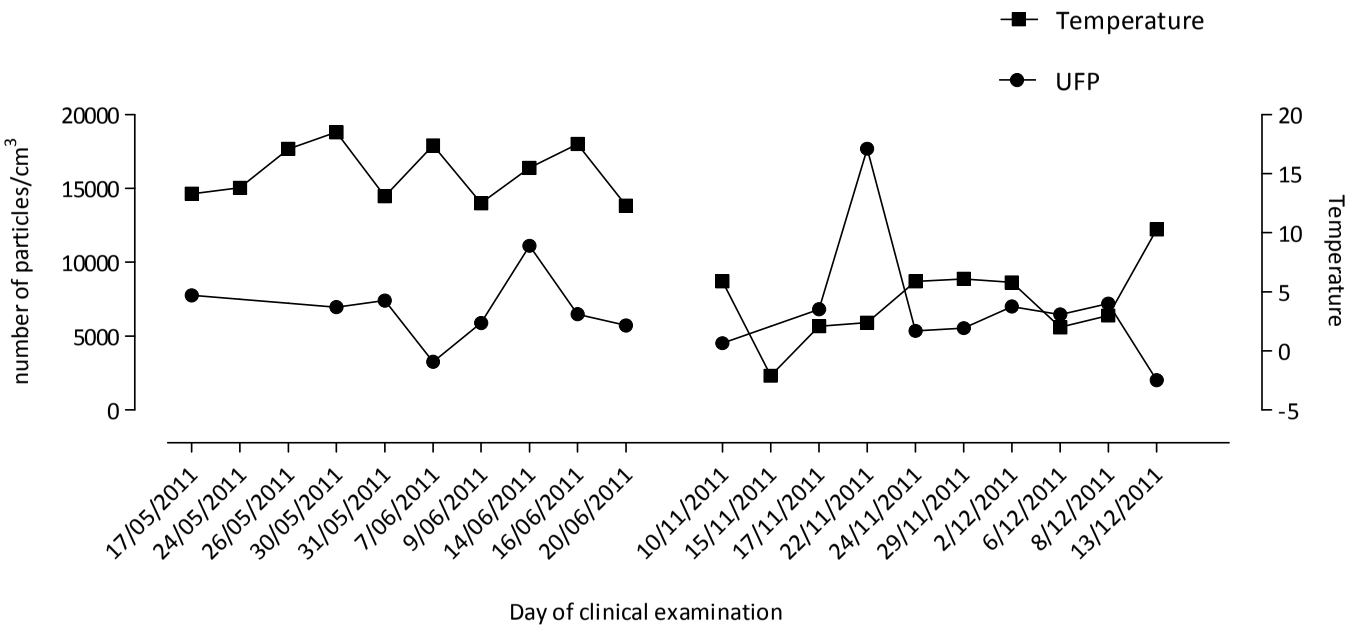

Figure 1: Concentration range of the accumulated UFP-fractions (left y-axis) and temperature (right y-axis) from 08.00 to 10.00 h AM on the day of clinical examination.

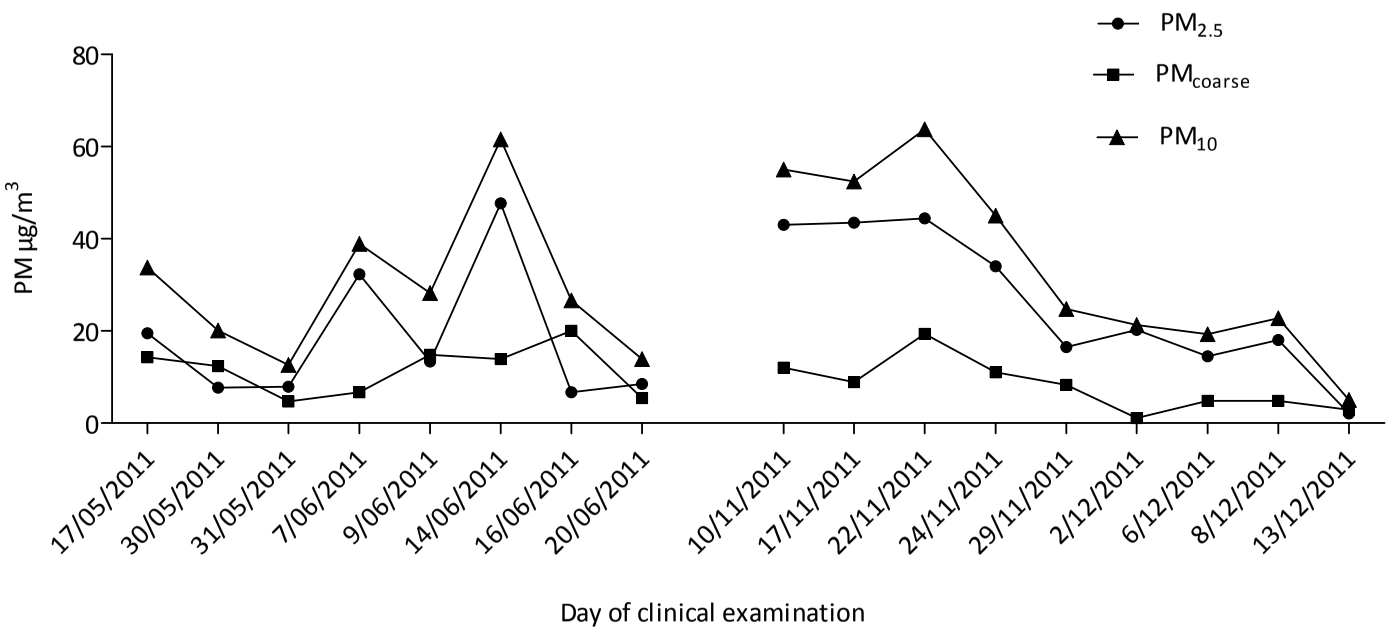

Figure 2: Concentration range for $PM_{2.5}$, PMcoarse and PM_{10} from 08.00 to 10.00 AM on the day of clinical examination.

Correcting for gender, age, height and weight of the child, parental education, neighborhood socio-economic status, fish consumption, heart rate, school, day of the week, season, wind speed, relative humidity, temperature and the interaction between season and temperature, systolic blood pressure was significantly associated with UFP fractions up to 100 nm, measured during the morning of clinical examination.

Systolic blood pressure increased by 6.35 mmHg (95% CI: 1.56 to 11.47, p=0.01) for each IQR increase in the smallest UFP fraction (20-30 nm). The corresponding effect sizes for the UFP fraction with a diameter of 30-50 nm, 50-70 nm and 70-100 nm were 1.18 mmHg (95%CI: 0.05 to 2.31; p=0.04), 0.92 mmHg (95%CI: -0.05 to 1.89; p=0.07) and 0.86 mmHg (95%CI: 0.05 to 1.68; p=0.04), respectively, while no effects were found for the fractions with a diameter larger than 100 nm, $PM_{2.5}$, PM_c and PM_{10}. For an IQR increase in the total UFP fraction, an increase of 0.79 mmHg (95%CI: 0.07 to 1.51; p=0.03) in systolic blood pressure was found. When the results were additionally adjusted for $PM_{2.5,}$ results were similar (figure 3). Diastolic blood pressure was not associated with either ultrafine particles nor with larger particulates (Supporting Table S2).

Additional adjustment for travel time to school or for residential distance to major roads gave similar results. Also exclusion of imputed UFP data and exclusion of days with high UFP concentrations did not alter the association between UFP and systolic blood pressure (Table 3). However, exclusion of days with low UFP concentrations were excluded from the analysis, the associations were no longer significant.

Finally, we investigated the acute response of exposure to lung inflammation. An IQR increase in the smallest UFP fraction was associated with a 24.2% increase in IL-1β (95%CI: 4.83 to 47.16, p=0.02) but no association appeared with $PM_{2.5}$ or PM_{10} (Table 4). The other ILs did not show a rapid increase with short-term increases in UFP (Data not shown).

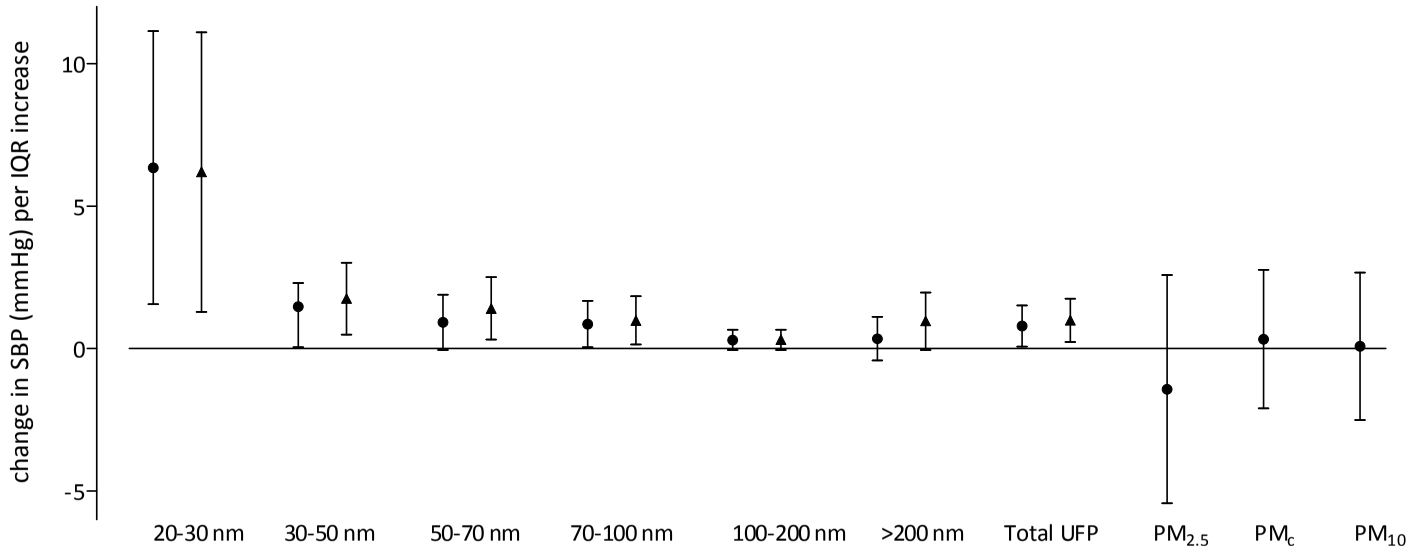

Figure 3: Association between systolic blood pressure and different UFP and PM fractions. Estimates (with 95% CI) represent the change in systolic blood pressure for an interquartile range (IQR) increase in the corresponding UFP or PM fraction. Dots show results adjusted for gender, age, height and weight of the child, parental education, neighborhood socio-economic status, fish consumption, heart rate, school, day of the week, season, wind speed, relative humidity, temperature on the morning of examination and the interaction between season and temperature. Triangles are additionally adjusted for $PM_{2.5}$ (only for the different UFP fractions)

Table 3: Sensitivity analysis, Estimates are given for an IQR increase in total UFP concentrations

	N	**Estimate**	**95% CI**	**p-value**
Model 1	220	0.79	0.07 to 1.51	0.03
Model 1+ travel time	211	0.78	0.03 to1.53	0.05
Model 1 + log distance to major roads	220	0.81	0.09 to 1.53	0.03
Exclusion of imputed UFP measurements	164	1.27	0.47 to 2.07	0.004
Exclusion of days with low UFP	182	0.42	-0.24 to 1.07	0.22
Exclusion of days with high UFP	193	1.96	0.32 to 3.61	0.02

Data were imputed on four days, exclusion of days with low total UFP (< 5000 particles /cm³) on two days, exclusion of days with high total UFP (> 10 000 particless/cm³) on two days

Table 4: Estimated % change in interleukin 1β (95% CI) per IQR increase in the corresponding UFP/PM fraction

UFP/PM Fraction	IQR	Estimate	95% CI	p-value
20-30 nm, #/cm^3	860	24.20	4.83 to 47.16	0.02
30-50 nm, #/cm^3	712	4.27	-0.56 to 9.35	0.09
50-70 nm, #/cm^3	540	3.79	-0.30 to 8.05	0.08
70-100 nm, #/cm^3	358	3.28	0.33 to 6.31	0.03
100-200 nm, #/cm^3	242	1.40	0.13 to 2.68	0.03
>200 nm, #/cm^3	136	1.98	-0.48 to 4.49	0.12
Total UFP, #/cm^3	1666	2.92	0.30 to 5.61	0.03
$PM_{2.5}$, μg/m^3	35	-6.28	-18.54 to 7.83	0.37
PM_c, μg/m^3	10	9.89	0.17 to 20.56	0.05
PM_{10}, μg/m^3	24	-1.33	-8.91 to 6.88	0.74

Regression coefficients calculated for a IQR increase in exposure. Models are adjusted for gender, age, height and weight of the child, parental education, neighborhood socio-economic status, fish consumption, school, day of the week, season, wind speed, relative humidity and temperature

DISCUSSION

In this study, we observed acute increases in children's systolic blood pressure in association with morning ambient ultrafine particles measured in the schools' playground. We only found significant results for particles with diameters smaller than 100 nm, while larger particles, $PM_{2.5}$ or PM_{10} were not significantly associated with blood pressure. To our knowledge, this is the first study examining the acute response of children's blood pressure in association with different size fractions of PM. In general, children might be more sensitive to air pollution due to their relatively higher ventilation rate and metabolic turnover, as well as by the fact that some of the organ systems including the immune system are still in development.[22] Furthermore, their physical behavior,

such as a greater physical activity, spending more time outdoors and their closer proximity to traffic exhaust emission sources compared with adults, might add to their vulnerability towards hypertensive effects of airborne particles.[22]

Because of their small size, UFP make up only a small fraction of the total $PM_{2.5}$ mass, even though they represent the largest actual number of particles within fine PM. Since UFP have a higher particle number concentration, the surface area is much higher and as such, carry large amounts of adsorbed or condensed toxic air pollutants which results in a different surface chemistry in comparison with larger particles.[23] Therefore, reductions in term of the mass of $PM_{2.5}$ do not necessarily results in lowering the risk of cardiovascular events.[24] This was also demonstrated in our study. We found that particle size is a determining factor in the effectiveness of particulate pollutants to cause rapid changes in blood pressure of 6-12-year olds, as we only observed an increase in children's blood pressure in association with exposure to particles smaller than 100 nm in diameter. Furthermore, the effect size decreased with increasing particle size. UFP and PM measurements were performed from 08.00h to 10.00h AM, while the clinical examination was organized between 09.00h AM and 01.00h PM. The mean UFP concentrations for the different fractions were highly correlated between the time frame of 08.00-08.30h AM and 09.30-10.00h AM (correlations >0.70). Therefore, the reported changes between 08.00-10.00h AM might also reflect the exposure later in the morning.

In contrast to some studies in adults[25] and pregnant women[7] or specific patients groups including adults with diabetes[6, 26] and elderly[9], we did not find rapid changes in blood pressure in association with $PM_{2.5}$ or PM_{10} concentrations.

We found a negative effect on blood pressure at temperatures above approximately 12°C and no associations at lower temperatures. These results are in line with effects reported in an article by Hampel et al. and Lanzinger et al. They found a 0.5% and 1 mmHg increase in systolic blood pressure for a 10°C and 1°C decrease in temperature, respectively [7, 27].

Unlike some other studies examining effects of air pollution, we did not observe changes in diastolic blood pressure in association with UFP fractions. It is

uncertain why exposures elicit a greater effect on systolic blood pressure rather than diastolic blood pressure. A possible reason is that systolic and diastolic blood pressures have different regulation pathways and can respond to environmental stimuli in a different way. While the main physiological role of systolic pressure is to force blood through the arteries during a heartbeat, which is responsive to the sympathetic nervous system and stress stimuli, the role of diastolic blood pressure is to provide perfusion of peripheral tissues during heart relaxation.[28]

The clinical significance of particulate-induced increases in blood pressure could be considerable. Childhood blood pressure is an important predictor of hypertension and cardiovascular disease later in life.[29-33] Although blood pressure is believed to be a complex trait, determined by numerous genetic, biological, behavioral, social and environmental factors, avoiding or removing potentially irreversible adverse factors as early as possible seems reasonable.[34] Indeed, repeated particle induced elevations in blood pressure also leads to repeated increases in arterial wall stress and may on the long-term result in chronically elevated pressures. Epidemiological evidence for a chronic increase in arterial stiffness in children due to traffic related air pollution, as exemplified by residential traffic related indicators, exists.[35]

Our current epidemiological observations in children are in line with human exposure studies. In a cross-over study, where subjects were exposed 2h to diesel exhaust, increases in systolic blood pressure were reported until 24h post-exposure. No effects on diastolic blood pressure were reported.[8] Further, a controlled experiment in healthy adults (aged 18-35 years) inhaling UFP for two hours showed changes in heart rate variability and loss of sympathovagal balance.[36] Existing evidence suggests that air pollution is able to trigger an acute autonomic imbalance, favoring sympathetic nerve activity causing smooth muscle contraction and thus vasoconstriction.[37] In a crossover experiment, systolic blood pressure was significantly lower during a 2h walk in Beijing, China, in subjects wearing a particulate-filter face mask than in subjects that were not protected by a face mask. Wearing the face mask was also associated with increased heart rate variability, which suggests that the rapid increase in blood pressure due to particle inhalation can be mediated through the autonomic

nervous system.[38] In other controlled studies, ultrafine carbon particles did not change blood pressure or heart rate variability but altered endothelial dysfunction or caused retinal vasoconstriction.[39-41]

Experimental evidence of intratracheally instilled UFP in hamsters showed that UFP can pass from the lungs into the blood circulation within minutes.[42] Due to specific characteristics (high surface area, particle number, metal and organic carbon content) of UFP, they may be transferred directly into the circulation and cause systemic inflammation and peripheral vascular oxidative stress resulting in reductions of nitric oxide, enhancing vasoconstriction and as such change blood pressure. Further, excess production of endothelin-1, a potent vasoconstrictor, after exposure to air pollution, can cause changes in blood pressure.[43] In animal models, plasma endothelin was up regulated after exposure to diesel exhaust and concentrated air particles.[44] These results were confirmed in an epidemiological setting where patients with metabolic syndrome and healthy volunteers showed an increase in plasma endothelin-1 concentrations three hours after diesel exhaust exposure.[45]

An association between PM and the development of pulmonary inflammation through pro-inflammatory cytokine induction, has been well documented.[46] Several ILs were measured in EBC, which is useful for detecting early lung inflammation.[47] We found evidence that some UFP fractions are able to cause elevations in IL-1β in EBC. IL-1β can be switched on by activated NF-ℵB as an early event of acute inflammatory response, which can subsequently lead to the production of other inflammatory cytokines.[48] Furthermore, the secretion of IL-1β could be considered as an early event in cardiovascular and respiratory illness due to its capacity to induce apoptosis, inhibit myofibroblasts differentiation and repress cell proliferation in rat lung fibroblasts.[49]

Our study has both strengths and limitations. Our study was limited in amount of repeated measurements and subjects because it was part of a larger biomonitoring program with a fixed design. The UFP concentrations did not differ significantly between the two periods (varied in both periods; see figure 1) consequently our results cannot be biased by adaptation towards the blood pressure measurements as variation in exposure was random and independent

of the first or second blood pressure reading. To account for diurnal variation in blood pressure, all children were examined on the same moment of the day. To reduce the effect of remaining variability, at least five blood pressure readings were taken after five minutes of rest in the sitting position and the first blood pressure measurement was excluded. Simonetti et al.[34] reported that parental smoking is an independent risk factor for children's blood pressure. In this regard, indoor smoking was an exclusion criteria, although this does not account for exposure to passive smoke elsewhere. As reported by Bilenko et al. and Liu et al., noise exposure might be a confounding factor in the association between air pollution and blood pressure.[11, 12] As we used a repeated measure design and the child was in both sampling periods examined on the same location and living at the same residential address at the different examinations, noise exposure is not a time-varying factor and therefore unlikely to bias our estimates on acute exposure. Additional adjustment for residential proximity to a major road, as a proxy for night time noise exposure, did not alter our association between systolic blood pressure and acute UFP exposure (table 3).

The major strength of the current study is the measurement of the different sized UFP and PM fractions in school playgrounds to reflect the acute exposure as accurately as possible.

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SUPPORTING INFORMATION

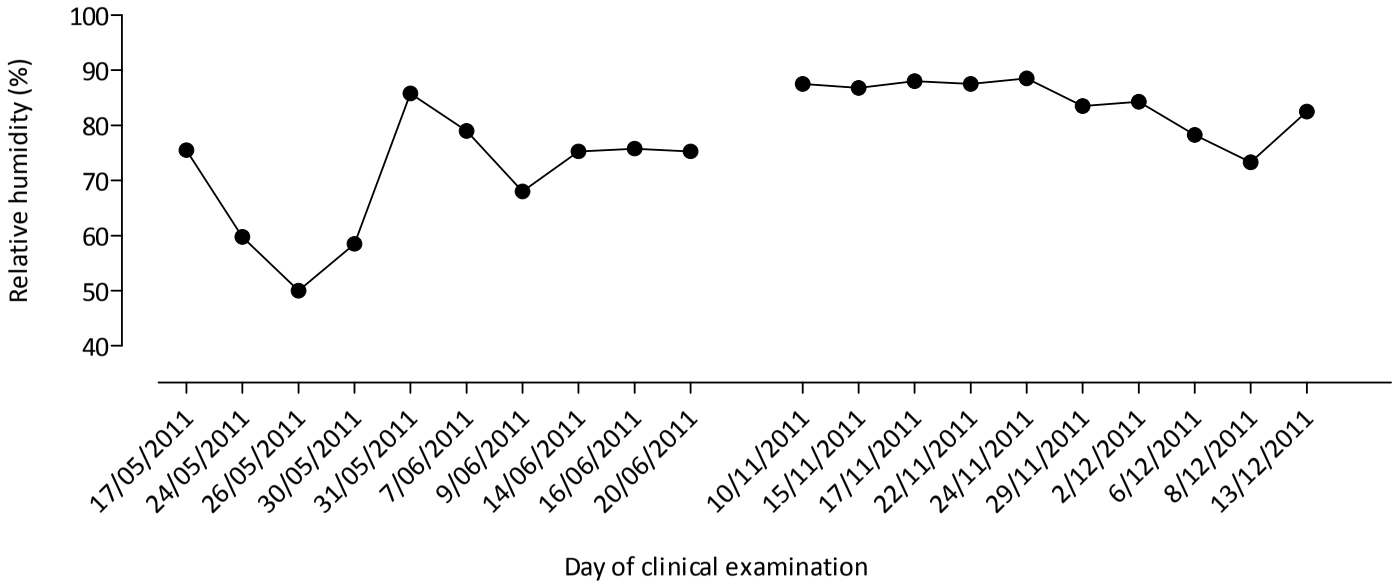

Figure S1: Relative humidity on the day of clinical examination

Table S1: Spearman Correlation coefficients between air pollution fractions

	Nano size (#/cm^3)							Coarse size (µg/m^3)		
	20-30 nm	30-50 nm	50-70 nm	70-100 nm	100-200 nm	> 200 nm	Total UFP	$PM_{2.5}$	PM_c	PM_{10}
Nano size (#/cm^3)										
20-30 nm	1									
30-50 nm	0.71**	1								
50-70 nm	0.38	0.80***	1							
70-100 nm	0.16	0.61**	0.87***	1						
100-200 nm	-0.11	0.24	0.54*	0.79***	1					
>200 nm	-0.21	0.29	0.52*	0.63**	0.73**	1				
Total UFP	0.62**	0.95***	0.93***	0.80***	0.40	0.40	1			

Coarse size									
$PM_{2.5}$	-0.13	0.32	0.22	0.44	0.53*	0.71**	1		
PM_c	0.11	0.22	0.36	0.36	0.39	0.41	0.23	1	
PM_{10}	-0.15	0.22	0.23	0.4	0.53*	0.69**	0.85***	0.66**	1

Levels of significance were indicated as *p<0.05, **p<0.01 and ***p<0.0001

Table S2: Change in diastolic blood pressure (in mmHg) per IQR increase in the corresponding UFP/PM fraction

UFP/PM Fraction	Estimate	95% CI	p-value
20-30 nm	0.58	-3.69 to 4.86	0.79
30-50 nm	-0.37	-1.41 to 0.66	0.48
50-70 nm	-0.26	-1.14 to 0.63	0.57
70-100 nm	0.01	-0.72 to 0.74	0.98
100-200 nm	0.05	-0.26 to 0.36	0.75
>200 nm (#/cm^3)	-0.27	-0.95 to 0.41	0.44
Total UFP	-0.09	-0.74 to 0.56	0.80
$PM_{2.5}$ (ug/m^3)	-2.80	-6.29 to 0.69	0.12
PM_c (ug/m^3)	0.03	-2.08 to 2.15	0.97
PM_{10} (ug/m^3)	-1.67	-3.93 to 0.58	0.15

CHAPTER 4

Molecular responses in the telomere-mitochondrial axis of ageing in the elderly: a candidate gene approach

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ABSTRACT

Both telomere length and mitochondrial function are accepted as reflective indices of ageing. Experimental evidence shows that telomere shortening induces mitochondrial dysfunction but so far studies in humans are scarce. Here, we investigated the association between relative leukocyte telomere length (LTL) and leukocyte mitochondrial DNA (mtDNA) content in elderly and explored intermediate mechanisms by determining the gene expression profile of candidate genes in the telomere-mitochondrial axis of ageing.

Among 166 non-smoking elderly, LTL, leukocyte mtDNA content and expression of candidate genes including sirtuin1 *(SIRT1*)*,* tumor protein p53 (*TP53*)*,* peroxisome proliferator-activated receptor γ-coactivator1α (*PPARGC1A*)*,* peroxisome proliferator-activated receptor γ-coactivator1β (*PPARGC1B*)*,* nuclear respiratory factor 1 (*NRF1*) and nuclear factor, erythroid 2 like 2 (*NFE2L2*), were analysed using a quantitative real time polymerase chain assay (qPCR). Statistical mediation analysis was used to investigate candidate genes as intermediate mechanisms of the telomere-mitochondrial axis of ageing.

Telomere length correlated with mtDNA content in our sample of elderly (partial r=0.23, p=0.005). *SIRT1* gene expression correlated positively with both telomere length (partial r=0.26, p=0.009) and mtDNA content (partial r=0.43, p<0.0001). *NRF1* showed significant correlations in the telomere-mitochondrial interactome. However these associations were found to be not independent of *SIRT1*. *SIRT1* gene expression was estimated to mediate 40% of the positive association between telomere length and mtDNA content.

The key finding of our study was that *SIRT1* expression plays a pivotal role in the telomere-mitochondrial interactome in non- smoking elderly.

INTRODUCTION

Senescence is caused by different biological processes and in this regard specifically telomeres, mitochondria and interactions between these are directly involved in the axis of cellular ageing.[1] Telomeres are nucleoprotein structures located at the end of chromosomes. They protect chromosomes from degradation, but in the absence of a compensatory elongating mechanism, telomeres become shorter with each cell division.[2] Excessive telomere shortening is an index of senescence, causes genomic instability and is associated with a higher risk of age-related diseases, such as cardiovascular disease[3] and cancer[4].

Mitochondria are involved in a variety of critical cell functions including oxidative energy production, programmed cell death, growth, and redox signalling. By-products of electron transfer reactions in mitochondria of aerobic cells result in the production of reactive oxygen species (ROS), e.g. superoxide and hydrogen peroxide.[5] Compared with nuclear DNA, mitochondrial DNA (mtDNA) is more vulnerable to damage due to the lack of protective histones and insufficient DNA repair capacity.[6] Among other mechanisms, maintenance of mitochondrial function has been suggested to be an important mechanism of extending lifespan, since decreased mitochondrial function, impaired ATP generation and increased ROS production are associated with ageing.[7] Mitochondria are linked to an array of metabolic and age-related diseases, including cancer[8], diabetes[9-11] and cardiovascular illness[12, 13]. Mitochondrial function, as exemplified by the mtDNA copy number, is inversely associated with cognition in elderly women.[14] Further, mtDNA copy number predicts all cause mortality: compared with the first quartile, the risk is 17% lower (95% CI: 29 to 2%) in subjects with mtDNA copy numbers above the 25th percentile.[14] Recently, Sahin *et al*. unveil a fascinating connection between the nuclear and mitochondrial ageing processes.[1] In telomere-deficient mice they show that the mitochondrial changes associated with ageing seem to be driven by the combined suppression of *peroxisome proliferator-activated receptor γ-coactivator1α (PPARGC1A)* and peroxisome proliferator-activated receptor γ-coactivator1β (*PPARGC1B*) and their downstream targets (nuclear respiratory factor 1, NRF1 and nuclear factor, erythroid 2 like 2, *NFE2L2*) through telomere dysfunction by a tumor protein p53

(*TP53*)-dependent repression.[1] Further evidence supporting the telomere-mitochondrial axis of ageing was observed in *SIRT1* knock-out mice. SIRT1 belongs to a group of highly conserved NAD^+-dependent protein deacetylases and functions as a metabolic sensor.[15] Increases in *SIRT1* were shown to stabilize *PPARGC1A* and, in turn, increase mitochondrial biogenesis and function.[15] Moreover, the positive impact of *SIRT1* may also arise from its deacetylation and the inactivation of *TP53*, which may attenuate checkpoint responses and depress *PPARGC1A* expression.[16]

To summarize, experimental evidence shows that telomere shortening induces mitochondrial dysfunction but, so far, studies in humans are scarce.[17] In the current study, we investigated the association between telomere length and mtDNA content in elderly and explored the expression of candidate genes (*SIRT1*, *TP53*, *PPARGC1A*, *PPARGC1B*, *NRF1*, *NFE2L2*) in the interactome of the telomere-mitochondrial axis of ageing.

METHODS

Study population

The total population (n = 3069) of the general medical practice in Genk (Belgium) is registered in the framework of a registration network for family practices in Flanders (INTEGO).[18] The study area is representative of the total population. Non-smoking men and women, aged 60 to 80 years, with no acute infection at enrollment and no history of malignancies, were selected in the southern region of Genk.[19, 20] Former smokers were only included if they quit smoking more than ten years before enrolment. Of those that were eligible, 166 persons were recruited by the general practitioner which resulted in a participation rate of 92%. For 41 persons (25%) no RNA samples could be collected. Questionnaires were administered through face-to-face interviews to assess lifestyle, profession, education, past smoking status, as well as information on age, weight and gender. Family income was given as net monthly overall family income and subdivided into low (<1500€), medium (1500€ - 3000€) and high (>3000€). Education was coded as low (primary school), medium (high school) and high (college or university). Self-reported physical activity was assessed by the number of times per week a person was engaged in

physical activities of more than 30 minutes (including walking, cycling, gardening,…). We gathered information on current and past use of medication from medical records of the medical practice. Anti-hypertensive medication reported included calcium antagonists, β-blockers, α-blockers and angiotensin-converting-enzyme inhibitors. Informed consent was obtained from all participants and the study was approved by the Ethical Committee of the East-Limburg Hospital (ZOL) in Belgium.

Sample Collection

Blood samples were collected from each participant in Vacutainer® Plus Plastic K2EDTA Tubes (BD, Franklin Lakes, NJ, USA) and PAXgene Blood RNA vacutainer tubes (Preanalytix, Qiagen, Hilden, GE) and analyzed in the clinical laboratory of the East Limburg hospital (ZOL) in Genk, Belgium. Blood cell counts and differential leukocyte counts were determined using an automated cell counter with flow differential (Cell Dyn 3500, Abbott Diagnostics, Abott Park, IL, USA). Blood glucose levels, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides were measured according to standard clinical procedures.

DNA analysis

Total DNA was extracted from white blood cells of the buffy coat using the MagMAX DNA Multi-Sample kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The yield (ng/µl) and purity ratios (A260/280 and A260/230) of the extracted DNA were determined with the Nanodrop spectrophotometer (ND-1000, Isogen Life Science, De Meern, NE). Extracted DNA was stored at -20°C until further use.

Measurements of leukocyte mitochondrial DNA content

Relative mtDNA content was determined using a quantitative real-time PCR (qPCR) assay by taking the ratio of two mitochondrial gene copy numbers (*MTF3212/R3319* and *MT-ND1*) to two single-copy nuclear control genes (*RPLP0* and *ACTB*). The forward and reverse primers for the mitochondrial genes were respectively 5'-CACCCAAGAACAGGGTTTGT-3' and 5'-TGGCCATGGGTATGTTGTTAA-3' for *MTF3212/3319*, and 5'-ATGGCCAACCTCCTACTCCT-3' and 5'-CTACAACGTTGGGGCCTTT-3' for *MT-ND1*. For the reference genes, the forward and reverse primers were respectively 5'-ACTCTTCCAGCCTTCCTTCC-3' and 5'-GGCAGGACTTAGCTTCCACA-3' for *ACTB*, and 5'-GGAATGTGGGCTTTGTGTTC-3' and 5'-CCCAATTGTCCCCTTACCTT-3' for *RPLP0*. Each sample was run in duplicate. A 10 µl PCR reaction contained Fast SYBR® Green I dye 2x (Applied biosystems, Lennik, BE) mastermix, forward (300 nM) and reverse (300 nM) primer and 12.5 ng DNA. All PCR-reactions were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The thermal cycling profile was similar for mtDNA and nuclear DNA: 20 sec at 95°C to activate the AmpliTaq Gold® DNA-polymerase, followed by 40 cycles of 1 sec at 95°C for denaturation and 20 sec at 60°C for annealing/extension. Each run was completed by a melting curve analysis to confirm the amplification specificity and absence of non-specific PCR products. Each PCR-plate contained six inter-run calibrators (IRCs) and two no-template controls (NTCs). After thermal cycling, raw data were collected and processed. Cq-values of the mitochondrial genes were normalized relative to the two reference genes using the qBase software (Biogazelle, Zwijnaarde, BE). The program uses modified software from the classic comparative CT method (ΔΔ CT) that takes into account multiple reference genes and uses inter-run calibration algorithms to correct for run-to-run differences [21]. Coefficient of variation (CV) within duplicates was 1.7% for mitochondrial genes and 1.6% for duplicates for the reference genes.

Measurements of leukocyte telomere length

Leukocyte telomere length was measured as telomere repeat copy number relative to two single gene copy numbers (T/S ratio) by a modified version of the previously described PCR-based telomere assay by Cawthon.[22]

The forward and reverse primer for the telomeres were 5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3' and 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'. The primers of the reference genes (*ACTB* and *RPLP0*) are the same as used for the mtDNA content measurement. The telomere reaction contained Fast SYBR® Green I dye 2x (Applied biosystems, Lennik, BE) mastermix, forward (100 nM) and reverse (900 nM) primer and 12.5 ng DNA. The telomere reactions were performed in triplicate. The thermal cycling profile for the telomere reaction consisted of following steps: 20 sec at 95°C, 2 cycles of 15 sec at 94° and 15 sec at 49° and 40 cycles of 15 sec at 94°, 10 sec at 62° and 15 sec at 74°. Amplification specificity and absence of primer dimers was confirmed by melting curve analysis at the end of each run. Each PCR-plate contained six IRCs and two NTCs. We also included two reference samples, one with relatively short telomeres and one with relatively long telomeres. Cq values of the telomere assay were normalized to two reference genes while taking into account run-to-run differences using qBase software (Biogazelle, Zwijnaarde, BE). CV within triplicates was 2.6% for telomeres and 1.6% for duplicates for the reference genes. CV for the exponentiated T/S ratio was less than 7%. Although this assay provides a relative measurement of telomere length, T/S ratios correlate well with absolute telomere lengths determined by Southern blot (r=0.9, n=20).

Gene expression analysis

Total RNA was extracted from PAXgene Blood RNA vacutainer tubes (Preanalytix, Qiagen, Hilden, GE) with the PAXgene Blood RNA kit (Preanalytix) with according to the manufacturer's instructions. cDNA was synthesized from 500 ng RNA using the Goscript Reverse Transcription System (Promega, Madison, Wi, USA) according to the manufacturer's instructions. A qPCR reaction was set up by adding 6.6 ng cDNA together with 5 µl Taqman Fast Advanced Master Mix (Life Technologies, Foster City, CA, USA) and 0.5 µl PrimeTimeTM assay (Integrated DNA Technologies, Coralville, IA, USA) in a final reaction volume of 10 µl. Cycling conditions for all transcripts were: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 sec at 95°C for 15 sec and 1 min at 60°C. We studied the gene expression of candidate genes within the telomere-*p53*-*PPARGC*-mitochondria axis (Table 1). Each PCR reaction was carried out in

triplicate and six IRCs and two NTCs were included in each 384-well plate. Amplification efficiencies of PrimeTime assays were determined by standard dilution series of a mixed sample, resulting in an efficiency between 90-110% for all assays and amplification specificity was confirmed by visualization on a 4% agarose gel. After thermal cycling, Cq values were collected and normalized to three reference genes, taking into account run-to-run differences with qBase software (Biogazelle, Zwijnaarde, BE). Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*), Acidic ribosomal phosphoprotein P0 (*RPLP0*) and hypoxanthine phosphoribosyltransferase 1 *(HPRT1*) were selected via GeNorm and Normfinder in a initial screen of nine reference genes as internal controls to normalize the data.

Statistical analysis

We used SAS software version 9.3 (SAS Institute Inc., Cary, NC) for database management and statistical analysis. Gene expression data, LTL and mtDNA content were log10 transformed to better approximate a normal distribution. We explored the correlation structure (partial Pearson correlation) between candidate genes and mtDNA content and LTL, while adjusting for sex, age, socio-economic status, past smoking status, white blood cell count and percentage of neutrophils. Mediation analysis was performed to explore the role of *SIRT1* as a mediator of the association between mtDNA content and LTL. This approach decomposes the total observed effect of telomere length on mtDNA content into a natural direct effect (NDE) and a natural indirect effect (NIE) that acts via the mediator of interest [23]. Mediation analysis requires a significant relation of the outcome to the mediator and a significant relation of the mediator to the exposure. Therefore, we only considered intermediate genes that satisfied all these assumptions (i.e. *SIRT1 and NRF1*). We assessed the IE of *SIRT1* with the classic causal step approach.[24]

A sensitivity analysis was performed by additionally adjusting for the following variables one at a time: HDL, LDL, CRP, blood glucose, myocardial infarction, asthma, COPD, systolic and diastolic blood pressure.

Table 1: Primer assay information of our selected candidate genes

Abbreviation	IDT Assay	Gene name	Ref seq number	Primer Efficiency, %	Exon Location	Amplicon Length, bp
HPRT_1	Hs.PT.39a.22214821	Hypoxanthine phosphoribosyltransferase 1	NM_000194	93	6-8	128
NRF1	Hs.PT.56a.3666627	Nuclear respiratory factor 1	NM_005011	106	12-13	102
NFE2L2	Hs.PT.56a.40946676.gs	Nuclear factor, erythroid 2-like 2	NM_006164	99	4-5	124
PPARGC1A	Hs.PT.56a.40982761	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	NM_013261	99	12-13	133
PPARGC1B	Hs.PT.56a.38577994	Peroxisome proliferator-activated receptor gamma, coactivator 1 beta	NM_133263	105	12-13	102
RPL0P	Hs.PT.56a.40434846	Acidic ribosomal phosphoprotein P0	NM_053275	101	7-8	146
SIRT1	Hs.PT.56a.40870995	Sirtuin 1	NM_001142498	94	9-10	133
TP53	Hs.PT.56a.39489752.g	Tumor protein p53	NM_001126114	90	16-16	146
YWHAZ	Hs.PT.39a.22214858	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	NM_003406	106	1-2	135

RESULTS

Study population characteristics

Descriptive characteristics of the study population are displayed in Table 1. Overall, the study population consisted of 166 elderly with a mean age (SD) of 70.6 (4.7) years. Body mass index (BMI) (SD) averaged 27.5 (3.7) kg/m^2 and 27.3 (5.1) kg/m^2 for men and women respectively. Of the 166 elderly, 89 (53.6%) were former smokers. The majority of former smokers were men (65.0%). Average (± SD) pack-years for former smokers was 19.0 ± 17.7 and 13.3 ± 12.5 for men and women, respectively. Telomere length (T/S ratio) decreased with 4.06% (95% CI: -8.06% to 0.61%; p=0.09) for each year increase in age. MtDNA content was not associated with age (p=0.41).

Associations between telomere length, mtDNA content and candidate genes of the telomere-mitochondrial axis

The association between telomere length, mtDNA content and the different candidate genes was first evaluated through a partial correlation matrix (Supporting table S1). All correlation coefficients were adjusted for sex, age, socio-economic status, past smoking status, white blood cell count and percentage of neutrophils. Telomere length was positively correlated with mtDNA content (partial r=0.23; p= 0.005). Telomere length was positively correlated with *SIRT1* (partial r= 0.26; p= 0.009) and *NRF1* (partial r= 0.20; p= 0.03), while telomere length was inversely correlated with *TP53* (r=-0.20; p=0.05). MtDNA content was strongly correlated with *SIRT1* (partial r=0.43; p<0.0001) and with *NRF1* (partial r=0.25; p=0.007). Further significant correlations were found between *SIRT1* and *NRF1* (partial r =0.76, p<0.0001), *SIRT1* and *PPARGC1A* (partial r = 0.28, p=0.005) and *NRF1* and *PPARGC1A* (partial r = 0.22, p=0.02). *SIRT1* was also inversely associated with *TP53* (partial r=-0.24, p=0.01) (Figure 1).

Table 2: Characteristics of the study population stratified by gender

Characteristics		**Men (n=77)**	**Women (n=89)**
Age, years		70.2 ± 5.1	70.8 ± 4.3
BMI, kg/m^2		27.5 ± 3.7	27.3 ± 5.1
Former smoker		58 (76%)	31 (35%)
Pack years		19 ± 17.7	13.3 ± 12.5
Education*			
	Low	28 (37%)	37 (42%)
	Middle	32 (42%)	31 (35%)
	High	16 (21%)	21 (24%)
Family income			
	Low	28 (37%)	45 (51%)
	Middle	46 (61%)	43 (48%)
	High	2 (3%)	1 (1%)
High density lipoprotein cholesterol, mg/dl		53.4 ± 18.2	66.3 ± 17.5
Low density lipoprotein cholesterol, mg/dl		112.9 ± 31.6	119.1 ± 36.3
High sensitivity C reactive protein, mg/dl		0.12 ± 0.3	0.13 ± 0.3
Glucose, mg/dl		106.1 ± 37.9	100 ± 29.5
Myocardial infarction		8 (10%)	6 (7 %)
COPD		10 (13%)	2 (2%)
Asthma		4 (5%)	2 (2%)
Systolic blood pressure		147.3 ± 18.2	142 ± 19.4
Diastolic blood pressure		90.6 ± 12.6	84.4 ± 11.6

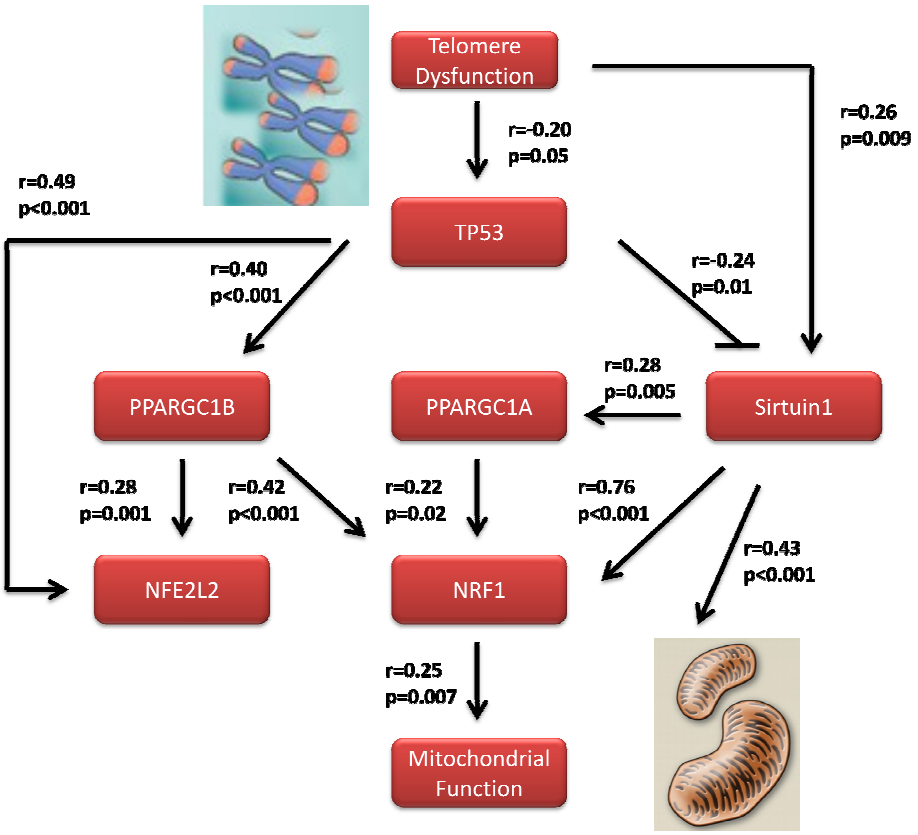

Figure 1: Partial Correlation of the studied candidate genes within the telomere-mitochondrial axis of ageing adjusted for adjusting for sex, age, socio-economic status, past smoking status, white blood cell count and percentage of neutrophils. Studied genes included *Sirtuin1 (SIRT1), tumor protein p53 (TP53), peroxisome proliferator-activated receptor γ-coactivator1α (PPARGC1A), peroxisome proliferator-activated receptor γ-coactivator1β (PPARGC1B), nuclear respiratory factor 1 (NRF1) and nuclear factor, erythroid 2 like 2 (NFE2L2).*

Mediating effects of candidate genes on the association between telomere length and mtDNA content

To formally test which genes could be intermediate targets on the association between telomere length and mtDNA content, mediation analysis was performed. As both *SIRT1* and *NRF1* were correlated with telomere length and mtDNA content, they fulfilled the criteria for mediation analysis [23]. However, the correlation between *NRF1* and our biomolecular targets of ageing (telomere length and mtDNA content) disappeared when we accounted for *SIRT1* expression. On the contrary, the correlation between *SIRT1* and telomere length and mtDNA content remained significant after additional correction for *NRF1*. Therefore, we only performed mediation analysis for *SIRT1*. Via mediation analysis, while adjusting for sex, age, socio-economic status, past smoking status, white blood cell count and percentage of neutrophils, *SIRT1* gene expression was estimated to mediate 40% (natural direct effect: 0.038, 95%CI: -0.11 to 0.088; natural indirect effect: 0.025, 95% CI: 0.003 to 0.048) of the positive association between telomere length and l mtDNA content (Figure 2).

In the sensitivity analysis, we additionally adjusted for HDL, LDL, CRP, blood glucose, myocardial infarction, asthma, COPD, systolic and diastolic blood pressure in separate models. The mediation-effect of *SIRT*1 remained significant and ranged from 36% to 46% (table 3).

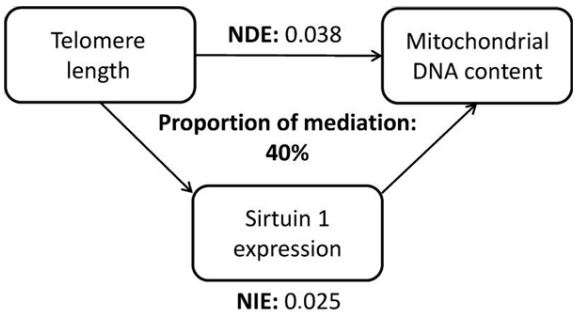

Figure 2: The proportion of the effect of telomere length on mitochondrial DNA content mediated through Sirtuin1 expression. NIE= indirect effect, NDE=direct effect.

Table 3: Sensitivity analysis: the proportion of the effect of telomere length on mitochondrial DNA content mediated through Sirtuin1 expression additionally adjusted for HDL, LDL, CRP, blood glucose, myocardial infarction, asthma, COPD, systolic and diastolic blood pressure in separate models.

	NDE		**NIE**		**TE**		**Proportion**
	Estimate	**p-value**	**Estimate**	**p-value**	**Estimate**	**p-value**	**Mediation %**
HDL	0.042	0.09	0.024	0.03	0.067	0.009	36
LDL	0.035	0.18	0.023	0.04	0.058	0.03	39
CRP	0.037	0.16	0.022	0.04	0.059	0.03	40
Glucose	0.033	0.19	0.024	0.03	0.057	0.03	42
Myocardial infarction	0.038	0.14	0.026	0.03	0.063	0.02	40
Asthma	0.034	0.19	0.029	0.02	0.063	0.02	46
COPD	0.038	0.14	0.025	0.03	0.064	0.015	40
Systolic BP	0.048	0.09	0.029	0.02	0.076	0.006	38
Diastolic BP	0.048	0.09	0.029	0.02	0.077	0.006	38

NDE: natural direct effect of (direct effect of telomere length on mitochondrial DNA content), NIE: natural indirect effect (indirect effect of telomere length on mitochondrial DNA content via Sirtuin1 expression), TE: Total effect

DISCUSSION

Mitochondrial dysfunction and telomere attrition are implicated in the ageing process. Moreover these hallmarks of ageing influence each other as telomere attrition leads to subsequent tp53-mediated repression of PPARGC1A and PPARGC1B, which can lead to reduced efficiency of mitochondria [1]. In 166 elderly, we studied the link between mtDNA and telomere length, two biological indicators of ageing, by determining the underlying expression profile of candidate genes. We found that *SIRT1* gene expression is a key determinant in the core axis of ageing between telomere length and mtDNA content. Our molecular epidemiological associations are in line with recent experimental work in rodents[1] and a human study[17] that showed an important link between telomere length and mtDNA content.

At the molecular level, when a critical short telomere length is reached (Hayflick limit) due to internal or environmental signals, *TP53* expression will be induced.[25] This DNA damage response can directly lead to mitochondrial and metabolic changes through the combined repression of the master regulators of mitochondrial biogenesis, *PPARGC1A* en *PPARGC1G*. In addition, it has been shown that *SIRT1* gene expression can inactivate *TP53*, and in turn, increase the expression of *PPARGC1A*.[1] In our study, we found a positive correlation of *PPARGC1B* and *TP53* but no correlation between *PPARGC1A* and *TP53*. We did, however, found evidence that *SIRT1* expression was inversely associated with *TP53* expression and through this way can induce expression of *PPARGC1A*. Furthermore, *SIRT1* expression was positively associated with both telomere length and mtDNA content, suggesting that *SIRT1* has a defining role in the telomere-mitochondria axis of ageing in elderly (Figure 2). This relationship holds true when we consider the results of our mediation analysis indicating that 40% of the effects of LTL on leukocyte mtDNA content was mediated by *SIRT1* expression and, therefore, strengthens the evidence of an intermediate mechanism of mtDNA alterations.

Overexpression of *SIRT1* in mice strains decreases incidence of several ageing related diseases, such as cardiovascular disease, metabolic disease and cancer.[15] The beneficial effects of SIRT1 in ageing were further marked by a

study in mice [26] where overexpression of *SIRT1* decreased the rate of telomere erosion associated with cell division and tissue ageing (i.e. liver and kidney) and *SIRT1* abrogation resulted in an increased telomere erosion. In response to excessive DNA damage and oxidative stress, p53 will be stabilized by acetylation and triggers apoptosis and cell cycle arrest. Conversely, deacetylation of p53 by SIRT1 inhibits its transcriptional activity and p53-dependent apoptosis. Overall, SIRT1 will promote cell survival. *SIRT1* can also promote mitochondrial biogenesis through deacetylation and activation of *PPARGC1A* and its downstream targets, nuclear respiratory factors *NRF1* and *NRF2*.[27] Here, we also showed the importance of *NRF1*, since the expression of this gene is associated with both telomere length and mtDNA content (Figure 1).

Furthermore, *NRF1* and *PPARGC1A* were correlated with *SIRT1* (Figure 1). *NRF1* can activate many genes involved in mitochondrial function and biogenesis.[28] The other studied genes including *PPARGC1B* and *NRF2* did not show an association with telomere length nor with mtDNA content. Although *NRF1* and *NRF2* are both stimulators of the expression of nuclear genes required for mitochondrial respiratory function, *NRF1* was shown to be the most potent, through its interaction with *PPARGC1A.*[27]

Since we found for the first time in an epidemiological context that mitochondrial DNA content is associated with telomere length with *SIRT1* as an important mediator, we further establish the importance of mitochondrial function in the axis of cellular ageing. Recently, we and others provided evidence that environmental exposures, such as particulate air pollution[29-31], polycyclic aromatic hydrocarbons[32] and benzene exposure[33] can influence mitochondrial function by altering mtDNA content. These environmental pollutants may decrease genome stability and contribute to the development of age-related diseases.[34] Furthermore, decreased mtDNA content has been associated with type II diabetes[9-11], soft cell sarcoma[35], ovarian cancer[36], breast cancer[37], gastric cancer[38], hepatocellular carcinoma[39] and renal cell carcinoma[40].

Some limitations of this study warrant consideration. Although our results were consistent after multiple adjustments, we cannot exclude that our findings were caused by non-studied genes playing a role in the mitochondrial and telomere

interactome. We used recently developed statistical methods on causal mediation[23] and our observations are in line with experimental evidence[1]. However epidemiological associations cannot prove the direction of association and it causality, though our statistical work plan was based on an a priori hypothesis on the direction of the association in experimental research.[1] Telomere length and mtDNA content were measured in a mixture of leukocytes. It was shown that neutrophils, which represent the majority of WBC and have a life span of approximately 10 h in blood lymphocytes, have only a 5% shorter telomere length compared to lymphocytes that have a longer turnover rate, suggesting that the effect of replicative history of the different cell types on telomere length may not be strong.[41] Changes in mtDNA content in human blood cells could also be attributed to platelet variation.[42] Platelet contamination increases mtDNA without an augmentation in nuclear DNA and affects mtDNA content.[43]. However, in a previous study by Janssen et al. 2012[29], mtDNA did not correlate with blood platelets, neutrophils, white blood cells or white blood cell/platelet ratio. Nevertheless, we adjusted our analysis for blood cell distribution.

We showed that *SIRT1* has a key role in the association between telomere length and mtDNA content in the elderly. Understanding candidate genes in the telomere-mitochondrial interaction in the elderly is important for the development of preventive and therapeutic measures supporting good health, prolonged active independence and a productive working life.

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SUPPORTING INFORMATION

Table S1: Pearson correlation matrix between LTL, mtDNA content and the studied candidate genes.

	Telomere length	mtDNA content	*SIRT1*	*PPARGC1A*	*NRF1*	*TP53*	*PPARGC1B*
mtDNA content	0.23**						
SIRT1	0.26**	0.43***					
PPARGC1A	0.09	0.11	0.28**				
NRF1	0.20*	0.25**	0.75***	0.22*			
TP53	-0.20*	0.03	-0.24*	0.05	-0.04		
PPARGC1B	-0.04	-0.03	0.09	0.11	0.42***	0.40***	
NFE2L2	-0.03	0.03	-0.13	0.06	-0.03	0.49***	0.28**

Level of significance was indicated as * p<0.05,**p<0.01 and ***p<0.001. *Sirtuin1 (SIRT1), tumor protein p53 (TP53), peroxisome proliferator-activated receptor γ-coactivator1α (PPARGC1A), peroxisome proliferator-activated receptor γ-coactivator1β (PPARGC1B), nuclear respiratory factor 1 (NRF1), nuclear factor, erythroid 2 like 2 (NFE2L2).*

CHAPTER 5

Biomolecular markers within the core axis of ageing and particulate air pollution in the elderly

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ABSTRACT

Telomere length and mitochondrial DNA (mtDNA) content are markers of aging and aging-related diseases. Exposure to airborne particulate matter (PM) results in adverse effects on the cardiovascular system, but the intermediate effects on markers of aging are currently inconclusive. Sirtuin1 (SIRT1) is an important determinant of longevity in humans that influences telomerase activity and might be an underlying mechanism in the etiology of PM-induced health effects.

The present study examines the association of long-term PM exposure with telomere length and mtDNA content in elderly and the proportion of the effect mediated by *SIRT1*.

Among 166 non-smoking elderly, telomere length, mtDNA content and gene expression of *SIRT1* and other candidate genes were measured using qPCR. Annual $PM_{2.5}$ exposure was calculated for each participant's home address using a Kriging interpolation model combined with a dispersion model.

Annual $PM_{2.5}$ concentrations ranged from 15 to 23 $\mu g/m^3$. A 5 $\mu g/m^3$ increment in annual $PM_{2.5}$ concentration was associated with a decrease of 16.8% (95% CI: -26.0% to -7.4%, p=0.0005) in telomere length and a decrease of 25.7% (95% CI: -35.2% to -16.2%, p<0.0001) in mtDNA content. Mediation analysis showed that *SIRT1* mediates respectively 19.5% and 22.5% of the effect of $PM_{2.5}$ on telomere length and mtDNA content. These observations are independent of gender, age, BMI, socio-economic status, statin use, past smoking status, WBC count and percentage of neutrophils.

The significant $PM_{2.5}$-induced effects on the telomere-SIRT1-mitochondrial axis of aging may play an important role in chronic health effects of $PM_{2.5}$.

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INTRODUCTION

Telomeres are complexes of hexameric repeats at the distal end of chromosomes where they provide stability and protection to the coding DNA. Telomere length declines with each cell division and thus can be considered as a marker of biological aging.[1] Excessive telomere shortening is an index of senescence, causing genomic instability and is associated with a higher risk of age-related diseases, such as cardiovascular disease[2] and cancer[3]. The natural erosion of telomeres associated with aging, can be accelerated through oxidative stress and inflammation induced by environmental factors[4, 5]. Shorter telomeres were found in smokers[6, 7] and in persons with higher exposure to traffic-related compounds such as benzene[8, 9]. Beside to telomeres, oxidative stress also targets mitochondria.[10] Maintenance of mitochondrial function has been suggested to be an important mechanism of extending lifespan whereas decreased mitochondrial function, impaired ATP generation and increased reactive oxygen species (ROS) production are associated with aging.[11] Recently, Sahin *et al*. unveiled a fascinating connection between the nuclear and mitochondrial aging processes.[12] Telomere-deficient mice, showed that the mitochondrial changes associated with aging seemed to be driven by the combined suppression of *peroxisome proliferator-activated receptor γ-coactivator1α (PPARGC1A)* and peroxisome proliferator-activated receptor γ-coactivator1β (*PPARGC1B*) and their downstream targets (*nuclear respiratory factor 1*, *NRF1* and *nuclear factor, erythroid 2 like 2*, *NFE2L2*) in response to telomere dysfunction through a tumor protein p53 (*TP53*)-dependent repression [12]. Further evidence supporting the telomere-mitochondrial axis of aging was observed in *sirtuin1* (*SIRT1*) knock-out mice. SIRT1 belongs to a group of highly conserved NAD^+-dependent protein deacetylases and functions as a metabolic sensor since the deacetylase activity is controlled by the cellular NAD^+/NADH ratio [13]. Increased *SIRT1* expression was shown to stabilize and, in turn, increased mitochondrial biogenesis and function.[13]

Multiple epidemiological studies showed associations between acute [14] or chronic exposure[15-17] to airborne particulate matter (PM) and adverse effects on the cardiovascular system, as well as increased incidence of cardiovascular related morbidity and mortality. Oxidative stress and systemic inflammation were

identified as a possible underlying mechanism for long-term exposure. Since oxidative stress is linked to both telomere attrition and mitochondrial DNA (mtDNA) damage, it is plausible that these markers of aging play a role in the chronic health effects of air pollution.[18]

To date, there is limited evidence that long-term exposure to air pollution can modulate telomere length and cause mtDNA damage[8] and mechanisms underlying this association have not been studied so far.

Here, we investigate in elderly whether biomolecular markers in the core axis of aging including telomere length and mitochondrial DNA are related to residential particulate air pollution exposure in elderly. Furthermore, starting from a candidate gene approach, we study possible mediators of the effects of air pollution on the telomere-mitochondrial interactome. We hypothesize that the effects of exposure to air pollution on mitochondrial DNA content can be mediated via telomere biology or SIRT1 expression.

METHODS

Study population

The total population (n = 3069) of a general medical practice in Genk (Belgium) is registered in the framework of a registration network for family practices in Flanders (INTEGO) that covers a representative part of the total Flemish population.[19] Non-smoking men and women, aged 60 to 80 years, with no acute infection at enrolment and no history of malignancies, were selected in the southern region of Genk.[20, 21]. Former smokers were only included if they stopped smoking more than ten years before enrolment. Of those that were eligible, 166 persons were recruited by their general practitioner, which resulted in a participation rate of 92%. For 41 persons (25%) no blood samples were available for RNA extraction. Questionnaires were administered through face-to-face interviews to collect information on lifestyle, profession, education, past smoking status, age, weight and gender. Family income was defined as net monthly overall family income and subdivided into low (<1500€), medium (1500€ - 3000€) and high (>3000€). Education was stratified as low (primary school), medium (high school) and high (college or university). Self-reported

physical activity was assessed by the number of times per week a person was engaged in physical activities of more than 30 minutes (including walking, cycling, gardening,…). We gathered information on current and past use of medication from medical records of the medical practice. Reported anti-hypertensive medication included calcium antagonists, β-blockers, α-blockers and angiotensin-converting-enzyme inhibitors. Informed consent was obtained from all participants and the study was approved by the Ethical Committee of the East-Limburg Hospital (ZOL) in Belgium.

Blood samples

Blood samples were collected for each participant in Vacutainer® Plus Plastic K2EDTA Tubes (BD, Franklin Lakes, NJ, USA) and PAXgene Blood RNA vacutainer tubes (Preanalytix, Qiagen, Hilden, GER). Blood cell counts and differential leukocyte counts were determined using an automated cell counter with flow differential (Cell Dyn 3500, Abbott Diagnostics, Abott Park, IL, USA). Blood glucose levels, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides were measured according to standard clinical procedures.

DNA analysis

Total DNA was extracted from white blood cells of the buffy coat using the MagMAX DNA Multi-Sample kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The yield (ng/µl) and purity ratios (A260/280 and A260/230) of the extracted DNA was determined with the Nanodrop spectrophotometer (ND-1000, Isogen Life Science, De Meern, NE). Extracted DNA was stored at -20°C until further use.

Measurements of leukocyte mitochondrial DNA content

Relative mtDNA content was determined using a quantitative real-time PCR (qPCR) assay by taking the ratio of two mitochondrial gene copy numbers (*MTF3212/R3319* and *MT-ND1*) to two single-copy nuclear control genes (*RPLP0* and *ACTB*). The forward and reverse primers for the mitochondrial genes were respectively 5'-CACCCAAGAACAGGGTTTGT-3' and 5'-TGGCCATGGGTATGTTGTTAA-3' for *MTF3212/3319*, and 5'-

ATGGCCAACCTCCTACTCCT-3' and 5'-CTACAACGTTGGGGCCTTT-3' for *MT-ND1*. For the reference genes, the forward and reverse primers were respectively 5'-ACTCTTCCAGCCTTCCTTCC-3' and 5'-GGCAGGACTTAGCTTCCACA-3' for *ACTB*, and 5'-GGAATGTGGGCTTTGTGTTC-3' and 5'-CCCAATTGTCCCCTTACCTT-3' for *RPLP0*. Each sample was run in duplicate. A 10 µl PCR reaction medium contained Fast SYBR® Green I dye 2x (Applied biosystems, Lennik, BEL) mastermix, forward (300 nM) and reverse (300 nM) primer and 12.5 ng DNA. All PCR-reactions were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The thermal cycling profile was similar for mtDNA and nuclear DNA: 20 sec at 95°C to activate the AmpliTaq Gold® DNA-polymerase, followed by 40 cycles of 1 sec at 95°C for denaturation and 20 sec at 60°C for annealing/extension. Each run was completed by a melting curve analysis to confirm the amplification specificity and absence of non-specific PCR products. Each PCR-plate contained six inter-run calibrators (IRCs) and two no-template controls (NTCs). After thermal cycling, raw data were collected and processed. Cq-values of the mitochondrial genes were normalized relative to the two reference genes using the qBase software (Biogazelle, Zwijnaarde, BE). The program uses modified software from the classic comparative delta-delta-Ct method that takes into account multiple reference genes and uses inter-run calibration algorithms to correct for run-to-run differences.[22] Coefficient of variation (CV) within triplicates was 1.7% for mitochondrial genes and 1.6% for duplicates for the reference genes.

Measurements of leukocyte telomere length

Telomere length was measured as telomere repeat copy number relative to two single gene copy numbers (T/S ratio) by a modified version of the previously described PCR-based telomere assay by Cawthon 23. The forward and reverse primer for the telomeres were 5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3' and 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'. The primers of the reference genes (ACTB and RPLP0) are the same as used for the mtDNA content measurement. The telomere reaction medium contained Fast SYBR® Green I dye 2x (Applied biosystems, Lennik, BE) mastermix, forward (100 nM) and reverse (900 nM) primer and 12.5 ng DNA. The telomere reactions were

performed in triplicate. The thermal cycling profile for the telomere reaction consisted of the following steps: 20 sec at 95°C, 2 cycles of 15 sec at 94°C and 15 sec at 49°C, and 40 cycles of 15 sec at 94°C, 10 sec at 62°C and 15 sec at 74°C. Amplification specificity and absence of primer dimers was confirmed by melting curve analysis at the end of each run. Each PCR-plate contained six IRCs and two NTCs. We also included two reference samples, one with relatively short telomeres and one with relatively long telomeres. Cq values of the telomere assay were normalized to two reference genes while taking into account run-to-run differences using qBase software (Biogazelle, Zwijnaarde, BEL). CV within triplicates was 2.6% for telomeres and 1.6% for duplicates for the reference genes. CV for the exponentiated T/S ratio was less than 7%.

Gene expression analysis

Total RNA was extracted from PAXgene Blood RNA vacutainer tubes (Preanalytix, Qiagen, Hilden, GER) with the PAXgene Blood RNA kit (Preanalytix) according to the manufacturer's instructions. For 41 persons RNA samples could not be collected. cDNA was synthesized from 500 ng RNA using the Goscript Reverse Transcription System (Promega, Madison, Wi, USA) according to the manufacturer's instructions. A qPCR reaction was set up by adding 6.6 ng cDNA together with 5 µl Taqman Fast Advanced Master Mix (Life Technologies, Foster City, CA, USA) and 0.5 µl PrimeTimeTM assay (Integrated DNA Technologies, Coralville, IA, USA) in a final reaction volume of 10 µl. Cycling conditions for all transcripts were: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 sec at 95°C and 1 min at 60°C. Overall, we studied the gene expression of candidate genes within the telomere-TP53-PPARGC1A-mitochondrial axis of aging (Table 1). Each qPCR reaction was carried out in triplicate and three NTCs and six IRCs were included in each 384-well plate. Amplification efficiencies of PrimeTime assays were determined by standard dilution series of a mixed sample, resulting in an efficiency between 90-110% for all assays and the amplification specificity was confirmed by visualization on a 4% agarose gel. After thermal cycling, Cq values were collected and normalized to three reference genes, taking into account run-to-run differences with qBase software (Biogazelle, Zwijnaarde, BEL). Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*), acidic ribosomal phosphoprotein P0 (*RPLP0*) and

hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) were selected via GeNorm and Normfinder as internal controls to normalize the data.

Exposure measurement

The annual exposure levels of $PM_{2.5}$ were calculated for each participant's home address using a spatial interpolation method (Kriging)[24, 25] combined with a dispersion model. The interpolation method uses pollution measurement data collected in the official fixed site monitoring network and land cover data obtained from satellite images (Corine land cover data set). The dispersion model described by Lefebvre[26, 27] uses the results from the interpolation method as background and superimposes the effects of industrial point sources and line sources from traffic to calculate the concentrations on a predefined grid. A correction for double counting is implied. This model chain provides hourly $PM_{2.5}$ values which are aggregated to annual means for each participant's home address.

Statistical analysis

Statistical analyses were conducted using the SAS statistical package, version 9.3 (SAS Institute, Cary, NC, USA). Gene expression data, telomere length and mtDNA content were logarithmically to better approximate a normal distribution. We used regression models to study the association between long-term exposure to particulate air pollution and aging related markers. We considered the following explanatory variables: gender, age, BMI, socio-economic status, statin use, past smoking status, white blood cell count and percentage of neutrophils.

Formal mediation analysis was performed to explore the role of *SIRT1* and telomere length as mediators of the association between exposure to particulate air pollution and markers of aging. This approach decomposes the total observed effect of exposure on markers of aging into a direct effect and an indirect effect that acts via the mediator of interest.[28] Mediation analysis requires a significant relation of the outcome to the exposure, a significant relation of the outcome to the mediator and a significant relation of the mediator to the exposure; as potential mediators. Therefore, we only analyzed

intermediates that satisfied all these assumptions (i.e. *SIRT1* and telomere length).

Table 1: Primer assay information of our selected candidate genes

Abbreviation	IDT Assay	Gene name	Ref seq number	Primer Efficiency, %	Exon Location	Amplicon Length, bp
HPRT_1	Hs.PT.39a.22214821	Hypoxanthine phosphoribosyltransferase 1	NM_000194	93	6-8	128
NRF1	Hs.PT.56a.3666627	Nuclear respiratory factor 1	NM_005011	106	12-13	102
NRF2	Hs.PT.56a.40946676.gs	Nuclear factor, erythroid 2-like 2	NM_006164	99	4-5	124
PGC-1α	Hs.PT.56a.40982761	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	NM_013261	99	12-13	133
PGC-1β	Hs.PT.56a.38577994	Peroxisome proliferator-activated receptor gamma, coactivator 1 beta	NM_133263	105	12-13	102
RPL0P	Hs.PT.56a.40434846	Acidic ribosomal phosphoprotein P0	NM_053275	101	7-8	146
SIRT1	Hs.PT.56a.40870995	Sirtuin 1	NM_001142498	94	9-10	133
TP53	Hs.PT.56a.39489752.g	Tumor protein p53	NM_001126114	90	16-16	146
YWHAZ	Hs.PT.39a.22214858	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	NM_003406	106	1-2	135

RESULTS

Study characteristics

Descriptive characteristics of the study population are displayed in Table 2. Overall, the study population consisted of 166 elderly with a mean age (SD) of 70.6 (4.7) years. BMI (SD) averaged 27.5 (3.7) kg/m^2 and 27.3 (5.1) kg/m^2 for men and women respectively. Of the 166 elderly, 89 (54%) were former smokers. The majority of former smokers were men (76%). Mean (SD) pack-year for former smokers was 19.0 (17.7) and 13.3 (12.5) for men and women, respectively. Annual mean (SD) $PM_{2.5}$ concentrations at the residence of study participants amounted to 20.8 (1.34) µg/m^3 and ranged from 15.7 µg/m^3 to 23.0 µg/m^3, and was considered as a proxy of long-term exposure to $PM_{2.5}$.

Association between air pollution indicators and markers of ageing

Telomere length (T/S ratio) was associated with a decrease of 4.06% (95% CI: -8.06% to 0.61%; p=0.09) for each year increase in age within the age range 60 to 80 years. MtDNA content was not associated with age (p=0.41). We selected candidate genes (table 1) based on their role in the mitochondrial – telomere axis.[12] Of these selected candidate genes, only *SIRT1* expression was significantly correlated with telomere length and mtDNA content, and is considered a marker of aging. Biomolecular markers of aging (mtDNA content, telomere length and *SIRT1* expression) were inversely associated with long-term $PM_{2.5}$ exposure (Figure 1). In multivariate models adjusted for gender, age, BMI, socio-economic status, statin use, past smoking status, white blood cell count and percentage of neutrophils, an annual 5 µg/m^3 increment in $PM_{2.5}$ exposure was associated with a relative decrease of 16.8% (95% CI: -26.0% to -7.4%, p=0.0005) in telomere length, a relative decrease of 25.7% (95% CI: -35.2% to -16.2%, p<0.0001) in mtDNA content, and a relative decrease of 17.3% (95% CI: -30.0% to -5.1%, p=0.006) in *SIRT1* expression (Figure 1).

Table 2: Characteristics of the study population stratified by gender

Characteristics		**Men (n=77)**	**Women (n=89)**
Age, years		70.2 ± 5.1	70.8 ± 4.3
BMI, kg/m^2		27.5 ± 3.7	27.3 ± 5.1
Former smoker		58 (76%)	31 (35%)
Pack years		19 ± 17.7	13.3 ± 12.5
Education*			
	Low	28 (37%)	37 (42%)
	Middle	32 (42%)	31 (35%)
	High	16 (21%)	21 (24%)
Family income			
	Low	28 (37%)	45 (51%)
	Middle	46 (61%)	43 (48%)
	High	2 (3%)	1 (1%)
High density lipoprotein cholesterol, mg/dl		53.4 ± 18.2	66.3 ± 17.5
Low density lipoprotein cholesterol, mg/dl		112.9 ± 31.6	119.1 ± 36.3
High sensitivity C reactive protein, mg/dl		0.12 ± 0.3	0.13 ± 0.3
Glucose, mg/dl		106.1 ± 37.9	100 ± 29.5
Myocardial infarction		8 (10%)	6 (7 %)
COPD		10 (13%)	2 (2%)
Asthma		4 (5%)	2 (2%)
Systolic blood pressure		147.3 ± 18.2	142 ± 19.4
Diastolic blood pressure		90.6 ± 12.6	84.4 ± 11.6

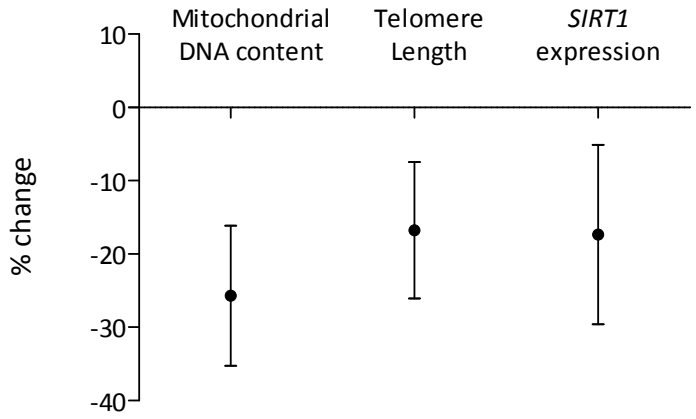

Figure 1: Relative difference in mitochondrial DNA content, telomere length and SIRT1 expression in association with an annual increase of 5 μg/m^3 in $PM_{2.5}$.

Mediation analysis

We performed mediation analysis to estimate the proportion of the effects of the exposure on mtDNA content that were mediated by telomere length and *SIRT1*. *SIRT1* gene expression satisfied the underlying assumptions for mediation analysis, since this marker showed significant associations with $PM_{2.5}$ exposure (Figure 1) as well as with mtDNA content and telomere length (Figure 2).

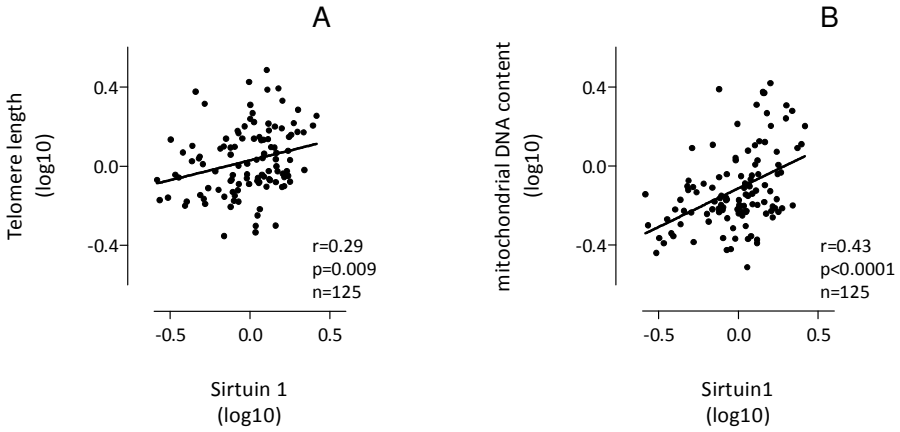

Figure 2: Association between (A) mitochondrial DNA content and (B) telomere and Sirtuin1 expression

Estimates of the proportion of mediation indicated that SIRT 1 mediates respectively 19.5% (95% CI: -8.1% to 22.5%, p=0.09) of the inverse association between $PM_{2.5}$ exposure and telomere length and 22.5% (95% CI:17.8% to 21.4% p=0.03) of the inverse association of $PM_{2.5}$ on mtDNA content (Figure 3).

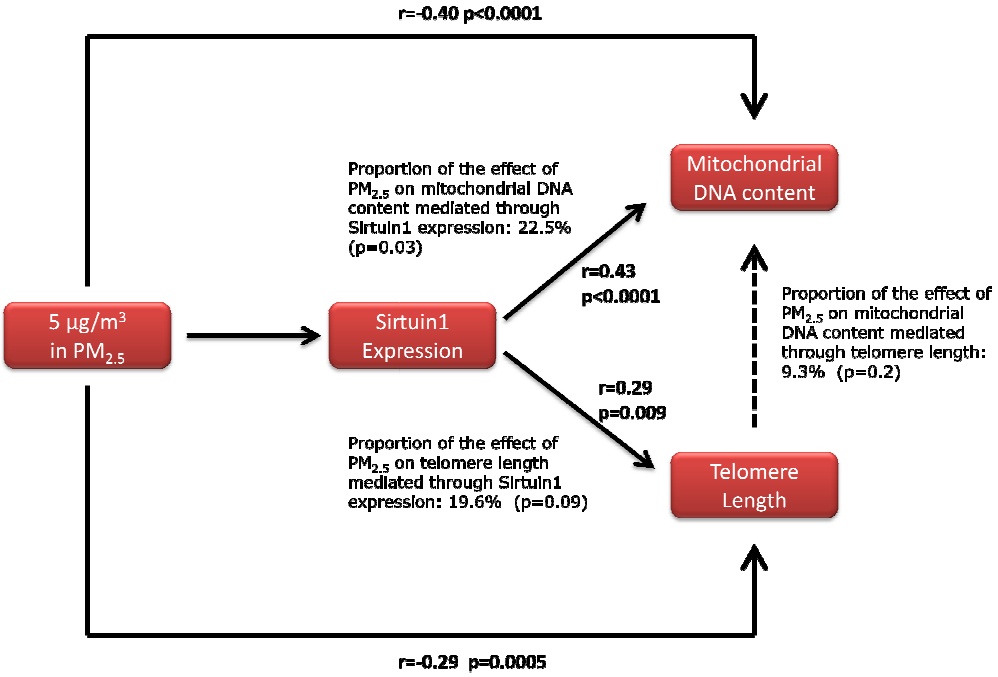

Figure 3: Correlation between different markers of aging and annual $PM_{2.5}$ ($\mu g/m^3$) exposure. The figure also displays the proportion of the effect of $PM_{2.5}$ ($\mu g/m^3$) exposure on mtDNA content and telomere length, mediated through *SIRT1* expression or telomere length.

DISCUSSION

The key finding of our study is that *SIRT1* and telomere length mediate the association between $PM_{2.5}$ exposure and lower mtDNA content, demonstrating that molecular targets in the core axis of ageing in the elderly are influenced by residential particulate air pollution.

Among 166 non-smoking elderly, we found that a 5 $\mu g/m^3$ increment in annual $PM_{2.5}$ was associated with a 16.8% decrease in telomere length and a 25.7% decrease in mtDNA content. Our results were independent of gender, age, BMI, socio-economic status and statin use. The public health significance of this effect

size on telomere length in our population can be illustrated by the fact that it corresponds to a chronological aging effect of 4 years. Furthermore, by use of formal mediation analysis, we provided evidence on the intermediate role of *SIRT1* expression on $PM_{2.5}$ induced changes in the telomere-mitochondrial axis of aging. These observations were done at concentrations below the EU $PM_{2.5}$ limit (25 μg/m^3) but above the US $PM_{2.5}$ limit (12 μg/m^3).

Extensive epidemiological studies support the associations between ambient air pollution and adverse health outcomes, including cardiovascular and respiratory disease, both with short-term[14, 29] and chronic exposure[30-33]. To date, studies examining the effects of PM exposure on telomere length reported different telomere responses after long-term or short-term exposure to PM. Short-term averages in ambient PM_{10} and $PM_{2.5}$ can lead to rapid increases in telomere length[34, 35], whereas annual concentration in black carbon was associated with a 8% decrease in telomere length.[9] Similar differences between short-term and chronic exposure to PM were also reported in association with mtDNA content. Hou et al. found an increase in mtDNA content in steelworkers[36] while they found a decrease in mtDNA content in office workers and truck drivers in association with short-term PM_{10} exposure[37]. A study in newborns[38] revealed that a 10 μg/m³ increment in PM_{10} exposure during the last trimester of pregnancy was associated with a 17.4% decrease in placental mtDNA content.

The biological mechanisms by which air pollution may cause adverse health outcomes is not completely understood but oxidative stress and inflammation are thought to be of importance. The ability of oxidative stress to damage nucleic acids provides a potential mechanism by which it could interfere with telomere DNA.[39] Due to their high content of guanine, telomeres are highly sensitive to ROS-induced damage.[40] Furthermore, telomeric DNA is ineffective in repairing single strand breaks. Accelerated shortening of telomeres, and as such, senescence of cells may be an important pathway by which oxidative stress may accelerate biological aging and the resultant development of aging-related morbidity, including cardiovascular disease.

Recent experimental[12, 41] and human studies[42] provide evidence of the relationship between mtDNA content and telomere length and form a platform

for age-related disease mechanisms.[18] Although a previous study has been performed in community-dwelling elderly women showing a positive correlation of leukocyte mitochondrial DNA copy number with telomere length[42], our study is the first to report the intermediate mechanisms of PM-induced mtDNA alterations by investigating the role of telomere length and *SIRT1* gene expression. The current accepted model proposes that DNA damage to telomeres activates several signaling pathways and alters *SIRT1* gene expression which lead to mitochondrial dysfunction [12, 41]. This hypothesis holds true when we consider the results of our mediation analysis indicating that the effect of $PM_{2.5}$ on mtDNA content was mediated by *SIRT1* expression. *SIRT1* is an important determinant of longevity in humans that influence telomerase activity[43] and also inactivates the 'guardian of the genome', *TP53*[44]. In addition, *SIRT1* activates *PPARGC1A*, a regulator of mitochondrial biogenesis.[45] Overexpression of *SIRT1* in mice strains was shown to reduce incidence of several aging related diseases, such as cardiovascular disease, metabolic disease and cancer.[46]

Some limitations of this study warrant consideration. Although the results were consistent after multiple adjustments, we cannot exclude that our findings were caused by some unknown factor that is associated with both mitochondrial function and telomere length. Although we used recently developed statistical methods on causal interference [28], they can never prove the biological direction of the findings. Nevertheless our observations are in line with experimental evidence.[12, 13] Secondly, telomere length and mtDNA content were measured in a mixture of leukocytes where cell composition differences between samples could influence our associations. However, neutrophils, which represent the majority of leukocytes, have a life span of approximately 10h in blood, have only a 5% shorter telomere length compared to lymphocytes that have a longer turnover rate, suggesting that the effect of replicative history of the different cell types on telomere length may not be strong.[47] In addition, changes in mtDNA content in human blood cells could also be attributed to platelet variation [48]. Platelet contamination increases mtDNA without an augmentation in nuclear DNA and affects mtDNA content.[49] However, in a previous study by Janssen[38], mtDNA did not correlate with blood platelets, neutrophils, white blood cells or

white blood cell/platelet ratio. Nevertheless, we adjusted our analysis for blood cell distribution.

Our findings on long-term $PM_{2.5}$ exposure indicate an inverse association on different biomarkers in the telomere-mitochondrial axis of aging. These observations further clarify the mechanism of action of air pollution. The paradigm that the telomere-mitochondrial core axis of aging plays a pivotal role in air pollution-induced health effects is novel. There is considerable public health concern because of the existence of high risk groups, including elderly. The public health significance of our reported effect size on telomere length can be illustrated by the fact that it corresponds to an aging effect of 4 years.

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CHAPTER 6

Decreased Mitochondrial DNA Content in Association with Exposure to Polycyclic Aromatic Hydrocarbons in House Dust during Wintertime: From a Population Enquiry to Cell Culture

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants that are formed in combustion processes. At the cellular level, exposure to PAHs causes oxidative stress and/or some of it congeners bind to DNA, which may interact with mitochondrial function. However, the influence of these pollutants on mitochondrial DNA (mtDNA) content remains largely unknown. We determined whether indoor exposure to PAHs is associated with mitochondrial damage as represented by blood mtDNA content.

Blood mtDNA content (ratio mitochondrial/nuclear DNA copy number) was determined by real-time qPCR in 46 persons, both in winter and summer. Indoor PAH exposure was estimated by measuring PAHs in sedimented house dust, including 6 volatile PAHs and 8 non-volatile PAHs. Biomarkers of oxidative stress at the level of DNA and lipid peroxidation were measured. In addition to the epidemiologic enquiry, we exposed human TK6 cells during 24 h at various concentrations (range: 0 to 500 µM) of benzo*(a)*pyrene and determined mtDNA content.

Mean blood mtDNA content averaged (± SD) 0.95 ± 0.185. The median PAH content amounted 554.1 ng/g dust (25^{th}-75^{th} percentile: 390.7-767.3) and 1385 ng/g dust (25^{th}-75^{th} percentile: 1000-1980) in winter for volatile and non-volatile PAHs respectively. Independent for gender, age, BMI and the consumption of grilled meat or fish, blood mtDNA content decreased by 9.85% (95% CI: -15.16 to -4.2; p=0.002) for each doubling of non-volatile PAH content in the house dust in winter. The corresponding estimate for volatile PAHs was -7.3% (95%CI: -13.71 to -0.42; p=0.04). Measurements of oxidative stress were not correlated with PAH exposure. During summer months no association was found between mtDNA content and PAH concentration. The ability of benzo(*a*)pyrene (range 0 µM to 500 µM) to lower mtDNA content was confirmed *in vitro* in human TK6 cells.

Based on these findings, mtDNA content can be a target of PAH toxicity in humans.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widespread pollutants, which are formed during incomplete combustion processes. Important sources of PAH exposure are motorized traffic and heating with fossil fuels. Some of the reactive metabolites of PAHs can bind to and damage macromolecules, including DNA. PAHs can induce oxidative stress indirectly trough cytochrome P450, epoxide hydrolase and dihydriodiol dehydrogenase, which results in the generation of quinones.[1] These redox active quinones are able to produce reactive oxygen species (ROS), thereby causing oxidative stress. It was shown that the PAHs and quinones, present on ultrafine particles, lead to functional and structural damage of the mitochondria, such as decreases in the mitochondrial membrane potential, either direct or secondary through oxidative damage.[2]

In normal conditions, ROS are generated in the mitochondria as metabolic by-products of the aerobic mechanism. ROS are continuously produced at the level of the mitochondrial electron transfer chain, where superoxide is produced by the one-electron reduction of oxygen.[3] Each mammalian cell contains approximately 200 to 2000 mitochondria, each carrying 2 to 10 copies of mitochondrial DNA. The mitochondrial DNA copy number is correlated with the amount and size of mitochondria.[4] Compared with nuclear DNA, mitochondrial DNA is more susceptible to damage because it lacks protective histones and has a diminished DNA repair capacity. As a result mitochondrial DNA has a high mutation rate and is particularly vulnerable to ROS-induced damage[5, 6], as well as to damage directly by adducts[7]. Initially, cells challenged with ROS synthesize more copies of their mitochondrial DNA and increase the number of mitochondria to compensate for the damage, resulting in a vicious circle of more ROS production from damaged mitochondria. However, in time, as defective mitochondria accumulate, bio-energetic and replicative function declines, leading to decreased or no synthesis of mitochondrial DNA.[8]

Surrogates of indoor PAH exposure have been measured in several environmental media, including air[9-11] and house dust[12-20]. Because PAHs can accumulate in carpets over years and decades, house dust PAH concentrations may be long-term predictors of indoor PAH exposure. According to Gevao et al.

2007, inadvertent dust ingestion is responsible for 11% of non-dietary total PAH exposure in adults and as much as 42% in young children.[19]

In the present study, we investigate the association of blood mitochondrial DNA content in association with indoor exposure to different PAH congeners. To establish a higher level of causality we performed, in addition to our study in humans, an *in vitro* experiment in which human cells were exposed to different concentrations of benzo*(a)*pyrene.

METHODS

Population study

Ethics Statement

Written informed consent was provided by all study participants in accordance with procedures approved by the Ethical Committee of the University of Antwerp (Reference nr. UA A09 22).

Subjects

We recruited two household members of 24 families. Only non-smokers, living in a smoke-free house and living for at least one year at their current residence, were included. The total population included 46 participants. A self-administered questionnaire was used to collect information on socio-economic status, lifestyle, general health, use of medication and the presence of risk factors. There were two sampling periods, one in winter (18 February- 2 March 2010) and one in spring/summer (5 June- 25 June 2010). In the period of dust sampling, blood- and urine samples were collected. Each participant was asked to complete a form with questions concerning diet and exposure during the 15 hours, prior to sampling.

Exposure measurement

The inhabitants of the residences collected sedimented house dust during 3 weeks using a vacuum cleaner. Also, blood and urine samples were collected within this period. The fine dust (ca. < 100 µg) in between two paper layers of the dust bag was extracted using soxhlet extraction. To eliminate interference of fats from food leftovers and products of biological origin, the PAH

fraction was separated from the fat fraction by gel permeation chromatography. Additional clean-up was performed using a combined silica/alumina column and the final sample was analysed with gas chromatography-mass spectrometry. Eight PAHs, typically considered as possible carcinogens, are described as non-volatile PAHs (benzo*(a)*anthracene, chrysene, benzo*(b)*fluoranthene, benzo*(k)*fluoranthene, benzo*(a)*pyrene, dibenzo*(a,h)*anthracene, benzo*(ghi)*perylene, *indeno(1,2,3-cd)*pyrene)[21, 22] and were measured in this study. Acenaphthylene, acenapthene, fluorine, phenanthrene, anthracene and the possible carcinogenic compound naphthalene, are specified as volatile PAHs . Fluoranthene and pyrene were also measured.

Biomarkers of oxidative damage

For analysis of plasma 15-F2T-isoprostane, a marker of lipid peroxidation, 500 µl plasma was collected in dark tubes containing 2 µl butylhydroxytoluene (5ng/ml 100% ethanol). Plasma 15F2T-isoprostane (pg/ml) was determined using an enzyme immuno-assay kit (Cayman Chemicals, Ann Arbor, MI, USA), according to the manufacturer's specifications. Urinary 8-deoxyhydroxyguanosine, a reflection of oxidative DNA damage, was measured with the New 8-OHdG Check from the Japan Institute for the control of aging (Gentaur, Kampenhout, BE).

Cell culture experiment

Benzo(a)pyrene was tested in the human lymphoblastoid cell line TK6. TK6 cells were purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK) and maintained in RPMI 1640 medium (Invitrogen, Merelbeke, BE) containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM l-glutamine at 5% CO_2 and 37°C. Prior to exposure, TK6 cells were seeded into 48 well-plate at a concentration of 0.5 x 10^6 cells/ well.

Benzo*(a)*pyrene (Sigma NV/SA, Bornem, BE) was dissolved in dimethyl sulfoxide (Sigma NV/SA, Bornem, BE). The solvent concentration (v/v) of the final culture volume was 1%. A mixture of S9 (1% v/v, Celsis, Neuss, GER) from human liver was added to the culture in half of the experiments. Cells were divided into 5 treatment groups (0.05, 0.5, 5, 50 and 500 µM benzo(a)pyrene), in either the

presence or absence of S9 mix. An exogenous NADPH-regenerating system (BD Biosciences, Erembodemgem BE) required by liver S9 for phase I oxidation was included in the experiments. Two solvent control groups (control S9−, control S9+) were also included. Because of the potential cytotoxicity of S9 preparations for cultured mammalian cells [23] a short term treatment (3 h) in the presence and absence of S9 was followed by removal of the test substance and a growth period of 21 h. Cells were exposed to benzo*(a)*pyrene in triplicates.

At the end of the exposure period, the cytotoxic response was evaluated with lactate dehydrogenase (LDH) activity assay as described previously [24]. The LDH measurement assesses membrane damage and is, therefore, indicative for cell death. We also counted the cells and assessed proportions of living and dead cells using a Countess™ Automated Cell Counter (Invitrogen, Carlsbad, CA).

Measurement of mitochondrial DNA content

For the population study, we collected whole blood in an heparin-coated vacutainer (BD, Franklin Lakes, NJ, USA) and total DNA was extracted from whole blood using a QIAamp DNA blood Maxi kit (QIAgen, Hilden, GER) following the manufacturer's instructions. For the TK6 cells, DNA was extracted with the QIAamp DNA mini kit (Qiagen, Hilden, GER). The concentration of extracted DNA was measured at 260 nm with the Nanodrop spectrophotometer (ND-1000, Isogen Life Science, De Meern, NE). Both DNA yield (ng/µl), which averaged 255 ng/µl for the blood samples and 82 ng/µl for the TK6 cells, and purity ratios A260/280 (range: 1.63-2.10) and A260/230 (range: 1.28-2.51) were determined. Extracted DNA was stored at -20°C until further use.

Relative mitochondrial DNA content was estimated using a quantitative real-time PCR (qPCR) assay by determining the ratio of the ND-1 mitochondrial gene to two nuclear reference genes (β-act and 36B4). Extracted genomic DNA was diluted to a final concentration of 5 ng/µl in RNase free water, prior to the qPCR runs. A 10 µl PCR reaction contained Fast SYBR® Green I dye 2x (Applied biosystems, Lennik, BE) mastermix, forward (10 µM) and reverse (10 µM) primer and 12.5 µg DNA. Primer details and efficiency are shown in table 7. All PCR-reactions were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The thermal cycling profile was

similar for mitochondrial DNA and nuclear DNA and consisted of following steps: 20 s at 95°C to activate the AmpliTaq Gold® DNA-polymerase, followed by 40 cycles of 1 s at 95°C for denaturation and 20 s at 60°C for annealing/extension. Each run was completed by a melting curve analysis to confirm the amplification specificity and absence of non-specific PCR products. Each PCR-plate contained four inter-run calibrators and two no-template controls. After thermal cycling, raw data were collected and processed. The range of the C_q values of the used genes and the no-template controls are given in table 1. C_q-values of the mitochondrial gene were normalized relative to the two reference genes using the qBase software (Biogazelle, Zwijnaarde, BE). The program uses modified software from the classic comparative C_T method ($\Delta\Delta$ C_T) that takes into account multiple reference genes and uses inter-run calibration algorithms to correct for run-to-run differences.[25]

Table 1: Characteristics of selected genes for qPCR

Gene symbol	**ND-1**	**β-act**	**36B4**
Nuclear/Mitochondrial	Mitochondrial	Nuclear	Nuclear
Accession number	NC_012920.1	NM_001101.3	NM_001002.3
Amplicon length (bp)	115	102	84
Forward 5' - 3'	ATGGCCAACCTCC TACTCCT	ACTCTTCCAGC CTTCCTTCC	GGAATGTGGGC TTTGTGTTC
Reverse 5' - 3 '	CTACAACGTTGGG GCCTTT	GGCAGGACTT AGCTTCCACA	CCCAATTGTCCC CTTACCTT
Primer efficiency (%)*	99.3 % - 104%	92 %-96.8 %	98% - 100.7 %
C_q range	16.36 - 18.75	23.47 – 25.45	23.21 – 24.93
Non template control range	32.42 - Undetermined	36.12 - Undetermined	35.92 - Undetermined

* Primer efficiency was determined in two different experiments

Mitochondrial NADH dehydrogenase 1 (ND-1; Beta actin (β-actin); Acidic ribosomal phosphoprotein P0 (36B4)

Statistical analysis

Statistical analyses were conducted using the SAS statistical package, version 9.2 (SAS Institute, Cary, NC, USA). The association between mitochondrial DNA content and PAH exposure was examined by mixed models using the MIXED procedure. Both mitochondrial DNA content and PAH exposure were log transformed and treated as continuous variables. Individuals nested within households were treated as a random factor and were included in the model to control for correlation between repeated observations at the level of the individual as well as the household. Models were adjusted for the following fixed effects: gender, age, body mass index and the consumption of grilled meat or fish during the last three days. Because the inclusion of an interaction term between season and the exposure revealed significant effect modification, we stratified analyses by season.

Cell culture data were analyzed using the non-parametric Kruskall-Wallis test. To study the trend over the exposure range, we used the Jonckheere-Terpstra test.

RESULTS

Population study

Characteristics of the study population

The median age of the 46 participants was 40 years (IQR: 32-47). Fifty two percent were men (table 2). Ten persons (22%) were former smokers. The participants had a mean (± SD) body mass index (BMI) of 24.2 kg/m^2 (± 3.3) in winter and 23.8 kg/m^2 (± 3.4) in summer (p=0.65) . The mean relative mitochondrial DNA content was similar for both seasons and amounted 0.954 in winter and 0.947 in summer (p=0.85, table 3).

Table 2: Characteristics of the study population

Characteristics		**median (IQR) or number (%)**
Male		24 (52.2%)
Age (y)		40 (32;47)
Former smokers		10 (22 %)
Heating source		
	Central heating	21 (84%)
	Electricity	4 (16%)
	Woodstove	8 (35%)

Data are presented as median (IQR= 25-75 percentile)or number (%)
Heating source data per household

Table 3: Characteristics of the study population stratified for winter and summer

Characteristics	**Mean ± SD or number (%)**		
	Winter	**Summer**	**p-value**
BMI	24.2 ± 3.3	23.8 ±3.4	0.65
Use of medication			
Bronchospasmolytica	3 (6.5%)	2 (4.4%)	0.99
H1 histamine antagonist	2 (4.4%)	2 (4.4%)	0.99
Glucocorticoïds	1 (2.2%)	0	0.99
Antihypertensives	2 (4.4%)	2 (4.4%)	0.99
Frequency consumption of grilled food*			
Daily	0	0	0.99
Weekly	2 (4.4%)	7 (15.2%)	0.16
Monthly	2 (4.4%)	20 (43.5%)	0.0001
Relative mitochondrial DNA content	0.954 ± 0.18	0.947 ± 0.19	0.85

Data are presented as number (%) or arithmetic mean ±SD
* Frequency of consumption of grilled food: data for one person, winter, missing

Exposure levels to PAHs

The non-volatile PAH en volatile PAH concentrations in house dust were higher in the winter period than in summer (table 4). The 3- and 4-ring phenanthrene, fluorene, pyrene (resp. ca. 12%, 17% and 12%) and the 4- and 5-ring structures chrysene and benzo*[b]*fluoranthene (resp. ca. 14% and 10%), made up the most important contribution to the measured PAH concentration in house dust (Figure 1). Sixteen participants (35%) lived in a house with regular use of a woodstove in winter. The volatile PAH, non-volatile PAH and benzo*(a)*pyrene concentration tended to be higher, although not significantly, in houses with this heating device compared to houses with another heating source in winter (data not shown).

Relative mitochondrial DNA content and indoor exposure to PAHs

Blood mitochondrial DNA content was similar in men and women (0.96 vs 0.99, p=0.48). We noticed significant season-by-PAH exposure interactions on mitochondrial DNA content. Therefore, we analyzed the data for summer and winter separately.

Table 4: Median amount (25th-75th percentile) of volatile and non-volatile PAHs and benzo*(a)*pyrene found in house dust (ng/g dust)

	Winter			**Summer**			
PAH	**Median**	**25th P**	**75th P**	**Median**	**25th P**	**75th P**	**p-value**
Volatile	554	390	767	446	311	655	0.04
Non-Volatile	1385	1000	1980	1258	733	1762	0.05
Benzo(a)pyrene	144	85	180	116	66	206	0.11

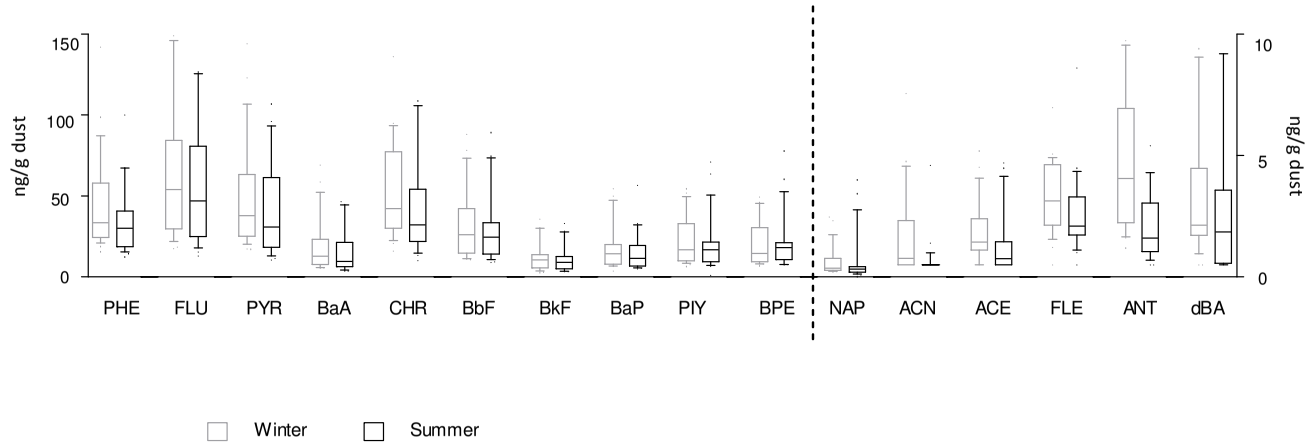

Figure 1: Median amount of each PAH-component in winter and in summer (ng/g dust). PHE, phenanthrene; FLU, fluoranthene; PYR, pyrene; BaA, benzo*(a)*anthracene; CHR, chrysene; BbF, benzo*(b)*fluoranthene; BkF, benzo(*k)*fluoranthene; BAP, benzo*(a)*pyrene; PIY, indenol*(1,2,3-cd)*pyrene; BPE, benzo*(g,h,i)*perylene; NAP, naphthalene; ACN, acenaphtylene; ACE, acenapthnene; FLE, fluorene; ANT, anthracene; dBA, dibenzo*(a,h)*anthracene

In winter, both before (figure 2) and after (table 5) cumulative adjustment for gender, age, BMI and the consumption of grilled meat or fish, blood mitochondrial DNA content was inversely and independently correlated with the indoor PAH dust concentration. When the analysis was repeated separately for non-volatile and volatile PAHs, we found that the effect was mostly attributed to non-volatile PAHs (table 5). We found a decrease of 9.85% (95% CI: -15.16% to -4.2%, p=0.002) in mitochondrial DNA content for each doubling in non-volatile PAH concentration and a 7.3% decrease (95% CI: -13.71% to -0.42%, p=0.04) for a doubling in volatile PAH, when adjusted for aforementioned variables. In addition we ran a separate multivariate analysis using benzo(*a*)pyrene dust exposure. Each doubling in benzo(*a*)pyrene exposuse was associated with a 7.18% decrease (95%CI: -11.82% to -2.3%, p= 0.007) in mitochondrial DNA content. In summer, with adjustments applied as before, blood mitochondrial DNA content was not associated with indoor PAH.

Biomarkers of oxidative DNA damage

No significant associations were found between plasma isoprostane levels and 8-deoxyhydroxyguanosine and PAHs in indoor dust (table 5).

Cell culture experiment

Human TK6 cells, exposed for 24h to different concentrations of benzo*(a)*pyrene (0 to 500 µM benzo*(a)*pyrene) showed a significant dose-dependent decrease in mitochondrial DNA content. The concentration of 0.5 µM and higher showed significant decreases in comparison with the control group and the cells exposed to the lowest concentration of 0.05 µM benzo*(a)*pyrene (figure 3). The Jonckheere-Terpstra test showed a significant decrease over the different exposures (p=0.0011). In S9 treated cells no decrease in mitochondrial DNA content was observed over the exposure range. TK6 cells viability and number of dead and living cells are given in table 7. Cell viability was comparable for all conditions, however, since the total number of living cells decreased with higher benzo*(a)*pyrene exposure, exposure to benzo*(a)*pyrene does not cause acute cytotoxicity but suppresses cell growth.

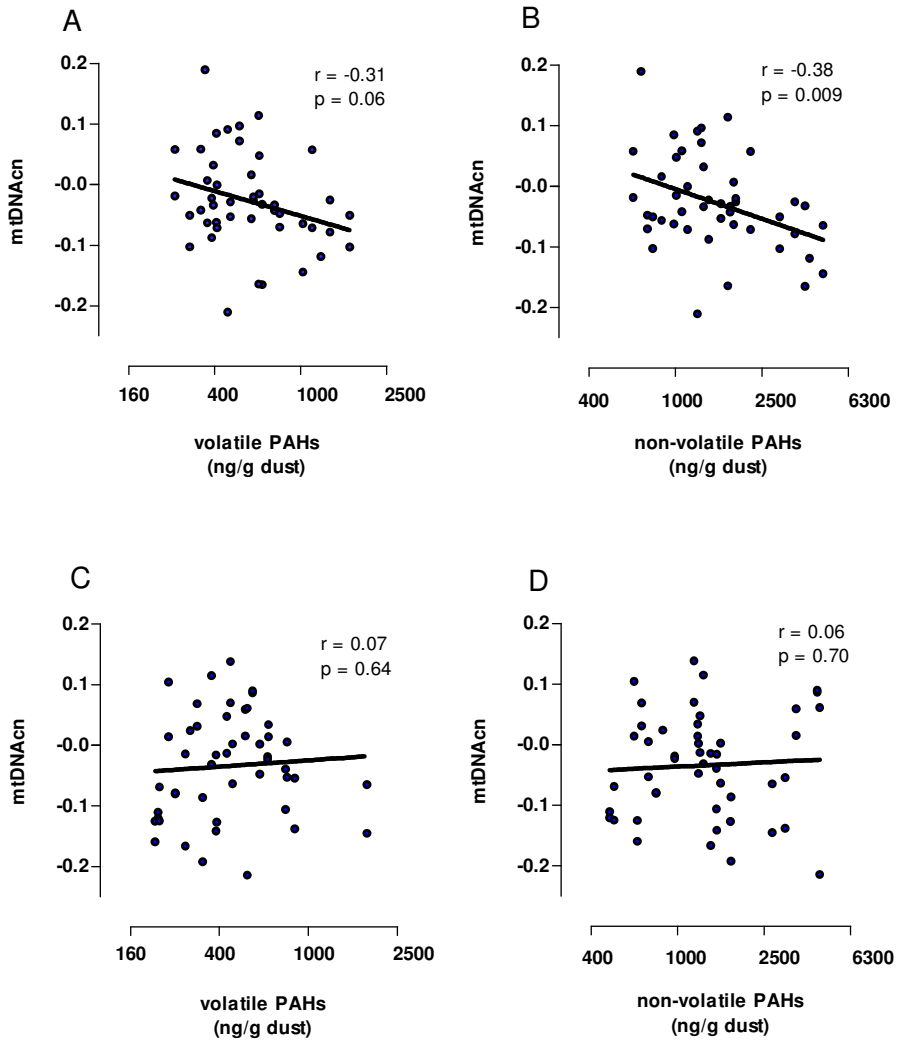

Figure 2: Association between mitochondrial DNA content and PAH exposure in winter and in summer. Four correlation plots are given, each indicating different PAH exposure, volatile PAHs in house dust in winter (A), non-volatile PAHs in house dust in winter (B), volatile PAHs in house dust in summer in (C) and non-volatile PAHs in house dust in summer in (D). Values of mitochondrial DNA content (mtDNAcn) are log transformed.

Table 5: Estimated change (95% CI) in plasma isoprostane and Urinary 8-hydroxydeoxyguanosine in association with PAH exposure and mitochondrial DNA (mtDNA) content

	Winter			**Summer**		
	Percentage*	**95% CI**	**p-value**	**Percentage***	**95%CI**	**p-value**
Plasma Isoprostane						
All PAHs	14.56	-9.04 to 38.18	0.23	-2.58	-36.06 to 30.90	0.88
Volatile PAHs	14.99	-9.68 to 39.67	0.24	-34.51	-73.7 to 4.68	0.09
Non-volatile PAHs	13.17	-9.17 to 35.51	0.25	10.23	-21.55 to 42	0.53
Urinary 8-hydroxydeoxyguanosine						
All PAHs	1.53	-4.92 to 7.98	0.64	0.09	-5.98 to 6.17	0.98
Volatile PAHs	6.11	-0.45 to 12.68	0.07	1.38	-5.88 to 8.64	0.71
Non-volatile PAHs	1.13	-4.94 to 7.20	0.72	0.0001	-5.83 to 5.83	0.99

* Percentage was calculated for each doubling in isoprostane and 8-hydroxydeoxyguanosine (based on a model with isoprostane, 8-hydroxydeoxyguanosine and log PAH)
Adjusted for gender, age, BMI and the consumption of grilled meat or fish. 8-hydroxydeoxyguanosine was additionally adjusted for urinary creatinine levels

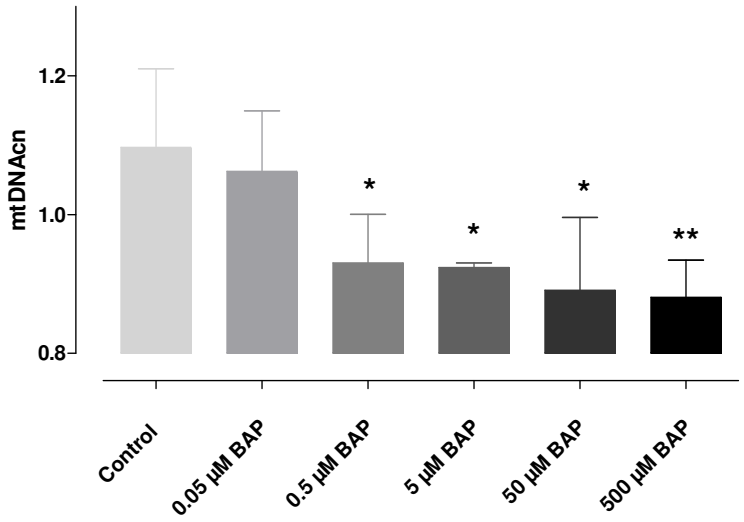

Figure 3: Mean mitochondrial DNA content in response to benzo*(a)*pyrene. Mean mitochondrial DNA (mtDNA) content of human TK6 cells exposed to 0; 0.05; 0.5; 5; 50 and 500 µM benzo*(a)*pyrene (BAP). Data are presented as mean ± SD; n = 3. *p < 0.05 vs control (0 µM BAP); **p < 0.01 vs. control (Analysis of variance: Kruskall-Wallis). Jonckheere-Terpstra test showed a significant (p=0.0011) decrease over the exposure range.

Table 7: TK6 cells viability and number of dead and living cells per exposure condition to benzo*(a)*pyrene (BAP)

	Cell viability (%)	**Number of cells ($x10^6$/ml)**	**Number of dead cells ($x10^4$/ml)**
Control	96	1.37	5.66
0.05 µM BAP	96	1.27	5.33
0.5 µM BAP	97	1.00	4.33
5 µM BAP	96	0.81	3.00
50 µM BAP	97	0.90	3.00
500 µM BAP	94	0.91	6.33

DISCUSSION

Mitochondrial DNA content correlates with the size and number of mitochondria, which have been shown to change under different energy demands, as well as different pathological conditions.[4] Experimental studies demonstrated that any genetic manipulation resulting in significantly decreased mitochondrial DNA content accelerates the ageing process and causes age-related disorders.[26] Therefore, mitochondrial DNA content might be an important and relevant target to study the effects of environmental exposures including PAHs. The key finding of our study is that mitochondrial DNA content is inversely associated with indoor exposure to PAHs in dust, including the group 1 carcinogen benzo*(a)*pyrene, in winter. This association was independent of gender, age, BMI and the consumption of grilled meat or fish. These findings were experimentally established in human TK6 cells, where mitochondrial DNA content also decreased in function of the benzo*(a)*pyrene concentration.

Our results are in line with a recent study on smoking.[27] In this study, a decrease in mitochondrial DNA content was observed in the lungs of smokers, which was attributed to the induced oxidative stress. Cigarette smoke contains many compounds, including PAHs (benzo*(a)*pyrene). In contrast to our results, others found that blood mitochondrial DNA content was increased in various occupational groups exposed to low benzene levels.[28] It has been suggested that the increased oxidative stress, caused by exposure to PAHs, has a dual influence on mitochondrial DNA content. Mild stress can stimulate mitochondrial DNA production and the number of mitochondria to fulfill in the respiratory needs of the cell and, as such, the cell will survive. But excessive oxidative stress may result in decreased or no synthesis of mitochondrial DNA due to the increasing abundance of defect mitochondria, eventually leading to cell senescence or cell death.[8] However, at the investigated concentrations no significant associations between PAHs in indoor dust and indicators of oxidative stress at the lipid level and nuclear DNA, as exemplified by plasma isoprostane level and urinary-8-hydroxydeoxyguanosine, respectively, were found.

Changes in the ratio between mitochondrial DNA content and nuclear DNA may be related to the development of multiple forms of disease. To date, many

studies reported increases or decreases in mitochondrial DNA content in response to endogenous or exogenous factors. Decreased mitochondrial DNA content has been shown in type II diabetes[29-31], soft cell sarcoma[32], ovarian cancer[33], breast cancer[34, 35], gastric cancer[36], hepatocellular carcinoma[37] and renal cell carcinoma[38]. Whether mitochondrial DNA content depletion has a role in tumorogenesis, is still under investigation, but is was demonstrated that extensive oxidative stress in cancer cells can cause changes in mitochondrial DNA content. This leads to alterations in mitochondrial gene expression and causes a deficiency in oxidative phosphorylation (OXPHOS).[39] A diminished OXPHOS activity was also demonstrated in aged tissues.[40] Mitochondrial mutations and simultaneous decreases in mitochondrial DNA content can ultimately lead to a detrimental cycle of further damage of the mitochondrial DNA but also genotoxic damage with rapid erosion and damage of telomeres.[26] As ageing is a complex process involving defects in various cellular components, we hypothesize that the changes observed on mitochondrial DNA content might be a relevant mechanism for cellular ageing by PAHs. Future studies on the link between PAH exposure and mitochondrial DNA content in association with effects observed in the nucleolus, such as telomere erosion, are necessary to elucidate the potential ageing pathways induced by exposure to PAHs.

We found no associations between indoor PAH exposure and mitochondrial DNA content in summer. This might be explained by several factors. PAH levels in the indoor environment during summer were lower. Residences with stove or open fire, tended (not significantly, probably due to the low number) to show a higher median indoor concentration of benzo*(a)*pyrene, non-volatile PAHs and volatile PAHs in winter. Also, during summer, people spend more time outdoors and their residences are in general more ventilated than in winter. Therefore, house dust in summer might be a less relevant PAH exposure marker.

Although our results were consistent after multiple adjustments, we cannot exclude that our associations obtained were due to residual confounding or were caused by some unknown factor that is associated with both mitochondrial function and exposure to PAHs. A clear limitation of this study is its small sample size. Since we analyzed DNA from whole blood we are capturing a mixture of leukocytes and the associations may be due to differences in white blood cell

subpopulations. Further, changes in mitochondrial DNA content in human blood cells could also be attributed to platelet variation.[41] Platelet contamination increases mitochondrial DNA without an augmentation in nuclear DNA and affects mitochondrial DNA content.[42] However, in a previous study by Janssen et al.[43], mitochondrial DNA did not correlate with blood platelets, neutrophils, white blood cells or white blood cell/platelet ratio. A clear strength is that our observational data in humans are confirmed by observations in cell cultures. We observed a dose dependent decrease in the mitochondrial DNA content of human lymphoblastoid TK 6 cells over a wide range of benzo*(a)*pyrene exposures.

In conclusion, PAH exposure in winter is associated with mitochondrial damage as exemplified by mitochondrial DNA content. Changes in mitochondrial DNA content might be an early target of PAH exposure. The potential health consequences of decreased mitochondrial DNA content and the role of PAHs in the ageing process must be further elucidated.

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CHAPTER 7

Cardiac autonomic dysfunction in children: impact of current air pollution exposure and protection by mitochondrial function

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ABSTRACT

Studies on the association between short-term exposure to ambient air pollution and heart rate variability (HRV) suggest that particulate matter (PM) exposure is associated with reductions in measures of HRV. However, evidence in children is lacking. Here we examine whether PM has an influence on children's HRV and evaluate whether mitochondrial DNA content reflects individual susceptibility.

We measured HRV in 67 children, aged 6-12 years on three different days (on average 46 days apart) during school time . Mitochondrial DNA content was measured in buccal cells collected at each visit. This resulted in a total number of 190 visits (2.8/child). We measured particulate exposure at the school's playground. Mixed-effect models were used to estimate the association between HRV and recent PM exposure and potential effect-modification by mitochondrial DNA content.

A 10 μg/m^3 increment in recent PM_{10} exposure was associated with a 2.34% (95%CI: -3.93% to -0.78%; p=0.003), 2.41% (95%CI: -4.57% to -0.28%; p=0.02), 4.40% (95%CI: -8.47% to -0.49%; p=0.03) and 4.37% (95%CI: -7.70% to -1.15%; p=0.008) decrease in the standard deviation of normal-to-normal intervals (SDNN), the square root of the mean squared difference of normal-to-normal intervals (rMSSD), high frequency (HF) power, and low frequency (LF) power, respectively. We found strong interaction between HRV and recent air pollution exposure in association to mitochondrial DNA content (p=0.03 for SDNN, p=0.08 for rMSSD, p=0.01 for HF and p=0.01 for LF), showing that decreases in mitochondrial function increases the effects of recent air pollution on HRV in children. HRV in children is associated with acute changes in particulate air pollution. Further, our data revealed that mitochondrial DNA content determines susceptibility to adverse autonomic effects of PM exposure in children.

INTRODUCTION

Heart rate variability (HRV) provides information on the autonomic nervous modulation of the cardiovascular system, with an altered function being on the trajectory to cardiovascular disease development. Short-term and long-term exposures to particulate matter (PM) air pollution contribute to cardiovascular morbidity and mortality.[1, 2] The association between HRV and PM has been investigated to clarify mechanisms underlying the increased risk of cardiovascular disease associated with PM exposure observed in multiple investigations. Our recent systematic review identified no studies in children on air pollution and HRV.[1] Findings in adults may not generalize to younger individuals, in view of the age-dependent differences in autonomic function [3-5] and the greater susceptibility of exposure to air pollution among children [6].

Air pollution effects on HRV have been suggested to be mediated through generation of oxidative species.[7] Findings from the Normative Aging Study indicated effect modification by glutathione-S-transferase M1 and methylenetetrahydrofolate reductase, and protective effects of statin use, dietary anti-oxidants and methyl nutrients on the PM-HRV relationship.[7-9] Because of the relevance of mitochondria for energy production and in oxidative stress generation[10], markers of mitochondrial dysfunction may help clarify the underlying mechanism and identify individuals at higher risk of exposure-related effects on HRV. We therefore investigated whether HRV in children is associated with recent exposure to PM air pollution and whether mitochondrial function, as exemplified, by mitochondrial DNA content modulates this association.

METHODS

Study Population

The ongoing COGNAC (COGNition and Air pollution in Children) study enrolls children, 9 to 12 years of age, recruited at primary schools in Flanders (Tienen, Zonhoven, Kiewit), Belgium. The study is specifically designed to investigate acute effects of air pollution on cognition. From January 2012 until February 2014, we recruited 334 children at three schools. HRV measurements were only implemented at the Kiewit school during the measuring campaign of

autumn and winter 2013-2014. Of the 200 eligible children, 67 participated in this study. We studied each child on three different examination days [on average 46 (range 29-64) days apart)]. This resulted in a total number of 190 visits (2.8 visits/per child). The clinical visits were scheduled on weekdays between 09.00 AM and 12.00 AM in the school. The children were examined on the same time point and weekday across the clinical visits. The parents were asked to fill out a questionnaire in order to obtain additional information on the child's residence, health status, ethnicity, smoking habits of the parents, means of transportation to and from the school and maternal and paternal socio-economic status. Written informed consent was requested from the parents and oral consent from the children. The study was approved by the Medical Ethics Committee of Hasselt University and the Hospital East-Limburg (ZOL).

Clinical measurements

HRV was measured for 10 minutes using the Zephyr Biomodule BH3 single-lead ECG monitor mounted on a Zephyr Biopatch (Procare, Groningen, NED) while the child was seated and had rested for 5 minutes. Standard deviation of normal-to-normal intervals (SDNN), the square root of the mean squared difference of normal-to-normal intervals (rMSSD), high frequency (HF) (0.15 to 0.4 Hz), and low frequency (LF) (0.04 to 0.15 Hz) were computed with a Fast Fourier transformation using the RHRV project [11]. For the analysis we selected 10 consecutive minutes of ECG measurements where artifacts were automatically removed and checked manually.

Air pollution monitoring

Current exposure to particulate matter pollution was measured at the school's playground. A portable laser-operated aerosol mass analyzer (Aerocet 531, Met One Instruments Inc, USA) was used to measure PM. This instrument measures $PM_{2.5}$ (particulate matter with an aerodynamic diameter less than 2.5 μm) and PM_{10} (particulate matter with an aerodynamic diameter less than 10 μm) mass concentrations in μg/m3. The instrument was previously calibrated against a European monitoring station (Flemish Environment Agency, Borgerhout, Antwerp, Belgium) [12]. Outdoor PM measurements were taken for 5 consecutive sampling periods of 2 min (10 min in total) approximately one hour

before clinical examination. We obtained daily average temperatures and daily average relative humidity from the Belgian Royal Meteorological Institute (Uccle, Brussels, BEL).

Mitochondrial DNA measurements

Buccal cells were collected at each clinical visit (3 total) using buccal swabs (SK2, Isohelix, Kent, GBR) All samples were stored at –80°C until further analysis. DNA from buccal cells was extracted using QIAamp DNA micro kit (QIAGEN, N.V. Venlo, NED). Relative mtDNA content was determined by a modified version of a quantitative real-time PCR (qPCR) assay, described previously.[13, 14] Briefly, mitochondrial DNA content was determined by taking the ratio of two mitochondrial gene copy numbers (MTF3212/3319 and MT-ND1) to one reference gene (36B4). Each sample was run in triplicate. A 10 µl PCR reaction mixture contained 1x Qiagen Quantitect Sybr Green Mastermix, 2 mM of dithiothreitol, forward (300 nM) and reverse (300 nM) primer and 12.5 ng DNA. All PCR-reactions were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The thermal cycling profile was similar for mitochondrial DNA and nuclear DNA: 20 sec at 95°C to activate the AmpliTaq Gold® DNA-polymerase, followed by 40 cycles of 1 sec at 95°C for denaturation and 20 sec at 60°C for annealing/extension.

Statistics

HRV measurements were logarithmically transformed to improve normality of the distribution. We performed pollutant exposure response analyses using mixed models that included random effects for each participant across the clinical examinations (SAS version 9.2, SAS Institute Inc, Cary, NC). This method allows each subject to serve as his or her own control over time and eliminates within-subject confounding by personal characteristics that do not change over time. We adjusted for the following confounders and covariates: gender, age, BMI and heart rate. In a sensitivity analysis, the models were additionally adjusted for apparent temperature. In order to account for possibly non-linear effects of temperature on HRV, we modeled this variable using restricted cubic splines with three knots. We tested the interaction between mtDNA content (continuous) and air pollution exposure for HRV. Because this

interaction was significant we stratified the analysis for mean mtDNA content using the 50th percentile of the distribution as a cut-off point to divide between low and high mtDNA content.

RESULTS

Descriptive statistics

Descriptive characteristics of the study population are given in Table 1. Mean (SD) height was 142.1 (9.6) cm and mean (SD) weight amounted to 34.9 (8.9) kg . We found gender-specific differences in HRV: all four parameters of HRV were higher for boys compared to girls.

Main effects of air pollution

Independent of gender, age, BMI and heart rate, we found that HRV measurements were inversely associated with current ambient air pollution. In all subjects, a 10 μg/m^3 increase in PM_{10} was significantly associated with SDNN (-2.31%; -3.94 to -0.70; p=0.005), rMSSD (-2.27%; -4.48 to -0.11; p=0.04), HF (-3.86%; -7.95 to 0.06, p=0.05) and LF (-4.20%; -7.60 to -0.91; p=0.01) (table 2). A 10 μg/m^3 increment in $PM_{2.5}$ was associated with a 3.51% decrease (-6.90 to -0.23; p=0.04) in SDNN (table 3). In a sensitivity analysis, we explored the role of apparent temperature in the PM-HRV association and found no significant confounding of apparent tempereature. Nevertheless when the model was additionally adjusted for apparent temperature, we found similar results for the different HRV parameters with PM_{10} and $PM_{2.5}$ (supplementary table S1).

Table 1: Characteristics of the study population

Characteristics	
Number of subjects	67
Girls	42 (66%)
Age, years	9.9 ± 1.2
Height, cm	142.1 ± 9.6
Weight, kg	34.9 ± 8.9
BMI, weigh/height^2	17.0 ± 2.8
Median SDNN, ms^2	63.1 (48.1;84.8)
Median rMSSD, ms^2	42.6 (29.9; 73.6)
Median HF power	221.4 (109.2; 570.9)
Median LF power	375.1 (204.1; 662.1)
Heart rate, beats/min	92.6 ± 14.9

Effect modification of mitochondrial DNA content

We noticed significant mtDNA content-by-air pollution interactions for HRV measures (table 2 and 3). When children were divided according to their mitochondrial status (i.e. low vs. high mitochondrial DNA content over the three examination periods), we found that the negative effect of PM_{10} and $PM_{2.5}$ was abrogated in subjects with higher than median mitochondrial DNA content. A 10 $\mu g/m^3$ increment in PM_{10} was associated with a 10.29% to 4.39% decrease in the different HRV-parameters in children with low mean mitochondrial DNA content, whereas no significant associations were observed in children with a mitochondrial DNA content above the median. Similarly, when stratified for mitochondrial DNA content, only children with low mitochondrial DNA content showed diminished HRV in the presence of higher $PM_{2.5}$ concentrations. The

modification of the PM_{10}-HRV association was statistically significant for SDNN, HF and LF, and borderline significant for rMSSD. The modification of the association between $PM_{2.5}$ and HRV was borderline significant for HF and LF.

Table 2: Change in HRV measurements in association with current PM_{10} exposure and effect-modification by mtDNA content

HRV component	Mitochondrial DNA content	% change	95% CI	p-value	p-interaction
Main Effect of PM_{10} on HRV, all subjects					
SDNN	All	-2.34	-3.93 to -0.78	0.003	/
rMSSD	All	-2.41	-4.57 to -0.28	0.03	/
HF	All	-4.40	-8.47 to -0.49	0.03	/
LF	All	-4.37	-7.70 to -1.15	0.008	/
Effect of PM_{10} on HRV, by mitochondrial DNA content[a]					
SDNN	< 1.05	-4.39	-6.90 to -1.95	0.0006	0.03
	≥ 1.05	-0.97	-3.00 to 1.02	0.33	
rMSSD	< 1.05	-4.72	-8.11 to -1.43	0.005	0.08
	≥ 1.05	-0.93	-3.71 to 1.79	0.50	
HF	< 1.05	-10.29	-17.00 to -3.96	0.001	0.01
	≥ 1.05	-0.60	-5.51 to 4.09	0.80	
LF	< 1.05	-8.55	-14.00 to -3.36	0.0014	0.01
	≥ 1.05	-1.59	-5.81 to 2.45	0.44	

Percent change (95%CI) in heart rate variability (HRV) for an increment of 10 $\mu g/m^3$ in PM_{10}, adjusted for gender, age, BMI and heart rate. [a]Mitochondrial status was dichotomized using the 50th percentile of the distribution as a cut-off point to divide between low (<1.05) and high (≥1.05) mtDNA content. HRV measurements were log transformed.

Table 3: Change in HRV measurements in association with current $PM_{2.5}$ exposure and effect-modification by mtDNA content

HRV component	Mitochondrial DNA content	% change	95% CI	p-value	p-interaction
Main Effect of PM_{10} on HRV, all subjects					
SDNN	All	-3.51	-6.90 to -0.23	0.04	/
rMSSD	All	-3.67	-8.28 to 0.74	0.1	/
HF	All	-4.86	-12.76 to 3.18	0.24	/
LF	All	-5.94	-13.14 to 0.79	0.08	/
Effect of PM_{10} on HRV, by mitochondrial DNA content[a]					
SDNN	< 1.05	-6.97	-12.88 to -1.37	0.01	0.15
	≥ 1.05	-1.84	-5.96 to 2.13	0.36	
rMSSD	< 1.05	-8.06	-15.98 to -0.69	0.03	0.21
	≥ 1.05	-1.75	-7.42 to 3.62	0.52	
HF	< 1.05	-13.83	-30.18 to 0.46	0.06	0.09
	≥ 1.05	-0.79	-10.83 to 8.34	0.87	
LF	< 1.05	-12.86	-26.07 to -1.03	0.03	0.05
	≥ 1.05	-2.53	-11.19 to 5.46	0.53	

Percent change (95%CI) in heart rate variability (HRV) for an increment of 10 $\mu g/m^3$ in $PM_{2.5}$, adjusted for gender, age, BMI and heart rate. [a]Mitochondrial status was dichotomized in low (<1.05) and high (≥1.05) mtDNA content using the 50th percentile of the distribution as a cut-off point HRV measurements were log transformed

DISCUSSION

This is the first time that data on HRV and current exposure to air pollution in children are described. All four measured HRV parameters in children were inversely associated with PM. rMSSD and HF both reflect parasympathetic cardial vagal tone while SDNN is an overall measure of changes in autonomic tone. LF variability is linked to both sympathetic and parasympathetic nervous system. Further, we demonstrated a significant modifying effect of mitochondrial status on the association between PM and HRV in schoolchildren, 9-12 years of age. Significant reductions in HRV with increasing PM concentrations were observed only in children with low mitochondrial DNA content. Our results suggest that mitochondrial status can influences the susceptibility to PM-linked effects.

Mitochondrial DNA content, an established marker of mitochondrial damage and dysfunction [15, 16], is thought to reflect the cumulative burden of oxidative stress, since mitochondria are the most important intracellular source of reactive oxygen species (ROS) and the major target of ROS. This study adds to other existing evidence that oxidative stress mediates the effects of air pollution on HRV. Drugs that modify oxidant defences may influence the susceptibility to particle-induced inflammatory or prooxidative responses. In addition to their cholesterol lowering effect, statins also have potent anti-inflammatory properties. The Normative Aging Study investigators found that the effect of PM_{10} on heart variability was confined to persons missing the allele for GSTM1 (lower oxidative stress defense), but the association was only apparent in those who were not under statin treatment.[9]

Further effect modification of anti-oxidative properties on the association between air pollution and HRV has been shown by dietary antioxidants and B vitamins, as well as u-3 polyunsaturated fatty acids.[8, 9, 17] These findings imply that pathways that decrease endogenous oxidative stress have a protective effect that alleviates reductions in HRV due to exposure of PM air pollution exposure.

So far, studies on the PM-HRV association focused on elderly and adult subjects.[1] Reliability of findings in adults may not be generalized to younger

individuals and children due to age-dependent differences in autonomic functioning. Further, various disease processes (myocardial infarction, diabetes, chronic obstructive pulmonary disease) as well as medication (β-blockers) alter autonomic control, and therefore change HRV. In view of our findings of PM-associated effects on HRV with current air pollution exposure in children, it should be pointed out that the association between HRV and air pollution exposure is not only seen in subjects with underlying diseases. We envision that children may be more susceptible to the adverse effects of air pollution due to their relatively higher ventilation rate and metabolic turnover, and the fact that organ systems including the immune system are still in development.[6] Furthermore, their lifestyle , such as a greater physical activity and spending more time outdoors may add to their vulnerability towards effects of airborne particles on the autonomic nervous system.[6]

Even during childhood, decreases in parasympathetic nervous activity are associated with cardiac autonomic neuropathy in diabetics with poor metabolic control[18], duration of diabetes[18], obesity[19] and elevated blood pressure[20].

Our study was rather limited in amount of repeated measurements and subjects. However, its major strength is the measurement of PM fractions at the school playgrounds to reflect the current exposure as accurately as possible.

Our findings, in accordance with observation in adults[1], support the hypothesis that exposure to PM air pollution leads to rapid changes in the autonomic nervous regulation of children. In addition, if confirmed, our findings might open new perspectives for risk stratification and individualized cardiovascular prevention early in life. Indeed, in children with low mitochondrial DNA content, air pollution might decrease the sympathetic predominance of HRV. Other lifestyle determinants acting via inflammatory or oxidative pathways and the modification by mitochondrial function must still be elucidated.

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SUPPORTING INFORMATION

Table S1: Change in HRV measurements in association with recent PM_{10} and $PM_{2.5}$ exposure, additionally adjusted for apparent temperature

HRV component	Exposure	% change	95% CI	p-value
SDNN	PM_{10}	-2.31	-3.94 to -0.70	0.005
rMSSD	PM_{10}	-2.27	-4.48 to -0.11	0.04
HF	PM_{10}	-3.86	-7.95 to 0.06	0.05
LF	PM_{10}	-4.20	-7.60 to -091	0.01
SDNN	$PM_{2.5}$	-3.36	-6.88 to 0.48	0.05
rMSSD	$PM_{2.5}$	-3.46	-8.21 to 1.09	0.1
HF	$PM_{2.5}$	-3.71	-12.49 to 4.39	0.4
LF	$PM_{2.5}$	-5.80	-13.23 to 1.15	0.1

CHAPTER 8

General discussion

SUMMARY OVERVIEW

Ageing is a complex physiological phenotype, responsive to both environmental and genetic factors. Inherent to ageing is the malfunction and decrease of biogenesis of mitochondria. In this doctoral dissertation, we focused on the role of mitochondrial DNA content as a possible intermediate between environmental exposure to ambient air pollution and cardiovascular function in the context of the 'exposome' concept.

Population and individual exposure levels to air pollution are associated with acute cardiovascular events such as myocardial infarction. However, the effect of air pollution on other cardiovascular parameters might be equally important to identify pathophysiological mechanisms by which air pollution may lead to cardiac mortality. Exposure to particulate matter has been associated with increases in blood pressure and HRV. By using a meta-analytical approach, we showed that HRV was inversely associated with a 10 $\mu g/m^3$ increase in all four parameters of HRV in a total population of 18 667 persons.

The systematic literature search of on heart rate variability (HRV) and PM, we identified, lead us to identify a knowledge gap since no studies in children on HRV and PM were reported. Further, so far, no short-term changes in blood pressure in association with PM_{10}, $PM_{2.5}$, nitrogen dioxide and ozone were found in school children.[1-3] In the context of the 'exposome' concept, we performed two studies in schoolchildren where the objective was to identify a possible relationship between short-term changes in PM and cardiovascular markers in childhood.

In the HEAPS (Health effects of air pollution in Antwerp schools) study, we found that blood pressure in children is association with different PM fractions. An interquartile range (860 particles/cm^3) increase in nano UFP fraction (20-30 nm) was associated with a 6.35 mmHg (95% CI: 1.56 to 11.14; p=0.01) increase in systolic blood pressure. The corresponding effect size for the total UFP fraction was 0.79 mmHg (95%CI: 0.07 to 1.51; p=0.03). In the COGNAC study, HRV was inversely associated with PM among 67 children, aged 6-12 years. A 10 $\mu g/m^3$ increment in recent PM_{10} exposure was associated with a 2.34% (95%CI:

-3.93% to -0.78%; p=0.003), 2.41% (95%CI: -4.57% to -0.28%; p=0.02), 4.40% (95%CI: -8.47% to -0.49%; p=0.03) and 4.37% (95%CI: -7.70% to -1.15%; p=0.008) decrease in SDNN, rMSSD, HF and LF, respectively. A 10 $\mu g/m^3$ increment in $PM_{2.5}$ was associated with a 3.51% (95% CI: -6.90% to -0.23%; p=0.04) decrease in LF.

Another objective of this dissertation was to explore the link between environmental exposure to mitochondrial DNA content, in order to find out whether mitochondrial DNA content could be an appropriate intermediate between exposure and outcome. In a population of adults, indoor PAH exposure was associated with decreases in mitochondrial DNA content in winter. The ability of PAHs to lower mtDNA content was confirmed *in vitro* in human TK6 cells by exposing the cells to benzo(*a*)pyrene (range 0 µM to 500 µM). During summer months, no association was found between mtDNA content and PAH concentration. In non-smoking elderly, a 5 µg increment in mean concentration of $PM_{2.5}$ was associated with a 10.6% decrease in leukocyte telomere length and an 11.4% decrease in mitochondrial DNA content. *SIRT1* expression was found to play a key role in the telomere-mitochondrial axis of ageing, indicating *SIRT1* being an underlying molecular target in ageing.

In the last part of this dissertation we explored the association between PM exposure, mtDNA content and cardiovascular function. Mitochondrial DNA content was identified as a susceptibility marker for the short-term effects of air pollution on HRV. Significant reductions in HRV with increasing PM concentrations were observed only in subjects with low mitochondrial DNA content, while no association with pollution was seen in subjects with high mitochondrial DNA content (figure 1).

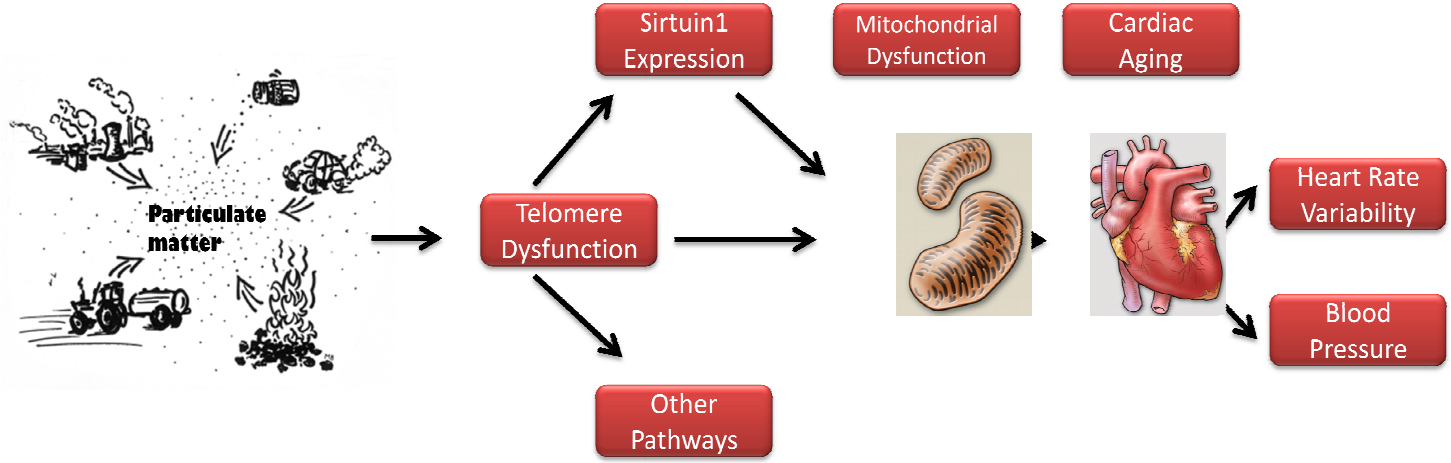

Figure 1: Overview of mitochondrial DNA content in association with PM and cardiovascular function

OBJECTIVE 1: MITOCHONDRIAL DNA CONTENT IN ASSOCIATION WITH ENVIRONMENTAL EXPOSURES

The exposome concept encompasses the totality of environmental exposures, from conception onwards. We and others provide evidence linking mitochondrial DNA content to different environmental exposures in different population segments. In this dissertation, we showed an inverse association of mitochondrial DNA content with PM and PAH exposure in both elderly and adults.[4]

Evidence on mtDNA content in relation to environmental exposures is still limited with inconsistent results as summarized table 1. A decrease in the mtDNA content in heavy smokers has been observed, which was attributed to the oxidative stress induced by smoking.[5] Moreover, Bouhours-Nouet et al.[6], showed that maternal smoking is associated with mtDNA depletion in placental tissue from newborns. Janssen et al.[7] showed that a 10 μg/m³ increase in PM_{10} exposure during the last month of pregnancy was associated with a 16.1% decrease in placental mtDNA content. We found a decrease in mtDNA content with PM in a population of elderly: an annual 5 $\mu g/m^3$ increase in $PM_{2.5}$ was associated with a relative decrease of 25.7% (95% CI: -35.2% to -16.2%, $p<0.0001$) in mitochondrial DNA content. Hou et al. found a significant negative association of 5- and 8-day means of ambient PM_{10} with mtDNA content in office workers and in truck drivers.[8] Personal elemental carbon levels, measured during the work day, were also associated with decreases in mtDNA content in the same population of office workers and truck drivers.[8]

Other studies on the mtDNA-smoking association showed an increase of mtDNA content in smokers.[9, 10] Two studies have demonstrated that individuals exposed to higher ambient benzene exhibited higher mtDNA content than participants with lower exposure.[11, 12] In our study on PAHs and mtDNA content, we found an inverse association between indoor PAH exposure and mtDNA content.[4] In a study of healthy steel workers in Northern Italy, personal PM_{10} and PM_1 were associated with and increased mtDNA content.[13] A group of Italian steel foundry workers with high exposure to metal-rich PM showed a dose–response relationship with increased mtDNA content on both the 1st and 4th day of the

study week, indicating that the correlations between PM exposure and mtDNA content were the result of a more protracted exposure to PM, rather than of the acute exposure between the two days.

As shown above, environmental exposures have been reported to result in both a decreased and increased mtDNA content. This may not just depend on the kind of pollutant but also on the dose and time point (short-term or chronic) and the tissue assessed. Increased oxidative stress has a dual influence on mitochondrial DNA content. At low doses oxidative stress appears to be able to stimulate mitochondrial biogenesis, but can result in a depletion at higher doses. This may be a related to of competing effects of ROS, resulting in both removal of damaged mitochondria[14] and induction of biogenesis.[15] Short-term depletion can also result in compensatory mechanisms over the long-term.[16]

All together, this evidence indicates that mitochondria are a relevant target of environmental contaminants in the context of the exposome, as effects of environmental factors on mtDNA content were shown in populations ranging from newborn to adults and elderly.

Table 1: Overview of epidemiological studies on mtDNA content and environmental exposures

Exposure	Exposure Time	Effect on mtDNA content	Tissue	Age Range	Author
Tobacco smoke					
Adults	Chronic	Increased	Saliva	18-94	Masayesva et al. 2006[17]
	Chronic	Increased light smokers Decreased heavy smokers	Lung tissue	16-85	Lee et al. 1998[5]
	Chronic	Increased	Buccal Cells Lymphocytes	19-87	Tan et al. 2008[9]
	Chronic	Increased	Whole Blood	50-69	Lynch et al. 2011[10]
Maternal	Chronic	Decreased	Placenta tissue	/	Bouhours-Nouet et al. 2005[6]
PM	Acute/Chronic	Increased	Whole Blood	27-55	Hou et al. 2010[13]
	Acute	Decreased	Whole Blood	31.9 ± 6.8	Hou et al. 2013[8]
	Chronic	Decreased	Placenta tissue		Janssen et al. 2012[18]
	Chronic	Decreased	Blood leukocytes	27-80	Pieters et al. (in preparation)
Elemental Carbon	Acute	Decreased	Whole Blood	31.9 ± 6.8	Hou et al. 2013[8]
Benzene	Acute	Increased	Whole Blood	30-55	Cargugno et al. 2012[11]
PAH	Chronic	Decreased	Whole Blood		Pieters et al. 2013[4]

OBJECTIVE 2: MITOCHONDRIAL DNA CONTENT IN ASSOCIATION WITH MOLECULAR AGEING NETWORKS

Maintenance of mitochondrial function has been suggested to be an important mechanism of extending lifespan, since decreased mitochondrial function, impaired ATP generation and increased ROS production are associated with ageing.[19] Mitochondria have been linked to an array of metabolic and age-related diseases, including cancer[20], diabetes[21-23] and cardiovascular illness[24, 25]. Mitochondrial function, as exemplified by the mtDNA copy number, is inversely associated with cognition in elderly women.[26] The integration of mitochondria into the 'core axis of ageing' is experimentally supported by the premature ageing conditions shared by telomere dysfunction as well as mutations or deficiencies in master regulators of mitochondrial biogenesis and function.[27] Evidence in rodents shows that the mitochondrial changes associated with ageing seem to be driven by the combined suppression of peroxisome proliferator-activated receptor γ-coactivator1α *(PPARGC1A*) and peroxisome proliferator-activated receptor γ-coactivator1β (*PPARGC1B*) and their downstream targets, nuclear respiratory factor 1 (*NRF1)* and nuclear factor, erythroid 2 like 2 *(NFE2L2*) through telomere dysfunction by a tumor protein p53 (*TP53*)-dependent repression.[27] Further evidence supporting the telomere-mitochondrial axis of ageing was observed in *SIRT1* knock-out mice. SIRT1 belongs to a group of highly conserved NAD^+-dependent protein deacetylases and it functions as a metabolic sensor [28]. Increases in *SIRT1* were shown to stabilize *PPARGC1α* and, in turn, increase mitochondrial biogenesis and function[28]. Moreover, the positive impact of *SIRT1* may also arise from its deacetylation and the inactivation of *p53*, which may attenuate checkpoint responses and depress *PPARGC1α* expression.[29]

Experimental evidence shows that telomere shortening induces mitochondrial dysfunction.[30] However, so far, studies in humans are scarce. In our study, a positive association was found between mtDNA content and telomere length, which is linked to disease progression and outcome, and which is also a marker of biological ageing.[31]

Human telomeres are complexes of hexameric repeats of DNA at the distal end of the chromosomes, where they provide stability and protection to the coding DNA. With each cell division there is an incomplete replication of the DNA-end whereby there is a loss of certain number of telomeric DNA pairs. This natural erosion of telomeres by chronological ageing can be accelerated or delayed by several genetic and environmental factors, and the interaction between them.

Environmental factors appear to overrate the contribution of the end-of-replication problem partly through oxidative stress. Airborne benzene and toluene, as indicators of traffic exposure in truck drivers, were associated with respectively a 6.4% and 6.2% decrease in telomere length. In comparison with control subjects (office workers), the effect of one year of ageing is a 0.5% decrease in telomere length.[32] Among 165 never smoking adults, an IQR increase in annual black carbon was associated with a 7.6% decrease in telomere length. For each year of within-subject change in age, telomere length declined on average 2.5%.[33] In our study on PM and the telomere-mitochondrial axis of ageing in elderly, we found that an annual increment of 5 $\mu g/m^3$ in $PM_{2.5}$ was equivalent to four years increase in age.

Further, we identified *SIRT1* as a target of environmental pollutants, since *SIRT1* expression mediates the PM-induced effects of mtDNA content. We found *SIRT1* to be a relevant molecular biomarker of ageing, through its association with telomere length, and in combination with mtDNA content. Beneficial effects of *SIRT1* were further reported in association with several ageing related diseases. *SIRT1* mediates the positive effects of calorie restriction which promotes cell survival through the deacetylation and activation of PPARGC1A which will turn on genes for fatty acid oxididation. In general *SIRT1* favours the oxidative metabolism in muscle, adipose tissue and liver, and insulin sensitivity.[34] The role of *SIRT1* in cancer is less clear. *SIRT1* extends the longevity of cells by downregulation of *P53*, but thereby it can also generate ca ncer in dividing cells. On the other hand, *SIRT1* functions as a tumor suppressor by its role in the DNA damage response and in the repair of DNA-double strand breaks.[35] *SIRT1* also has a role in suppression of inflammation, as it represses nuclear factor kappa-light-chain-enhancer of activated B cells (*NFκB)*, which controls the activity of genes involved in apoptosis, cell senescence, inflammation and

immunity. By dampening the activation of *NFκB*,[35] *SIRT1* augments apoptosis in response to tumor necrosis factor α. Overall, this evidence indicates a key role for *SIRT1* in the biological ageing process.

OBJECTIVE 3: MITOCHONDRIAL DNA CONTENT AS AN INTERMEDIATE BETWEEN EXPOSURE AND OUTCOME

Both environmental and genetic factors influence mtDNA homeostasis. A twin study by Xing et al.[36] demonstrated high heritability of mitochondrial DNA content. Genetic host factors that modify pathophysiological effects of particles, as well as livestyle factors, may play an important role in predicting susceptibility to air pollution. In the previous section, we showed that PM can influence mitochondrial DNA content. Therefore, we aimed to determine whether mitochondrial DNA content is an underlying susceptibility marker or mediator of PM-induced effects.

The importance of mtDNA content as a mediator between exposure and outcome was demonstrated by Clemente et al. (data not published). We showed that a decreased placental mtDNA content in early life was associated with *in utero* PM_{10} and NO_2 exposure. Further, placental mtDNA content was significantly and positively associated with birth weight, while birth weight decreased significantly with 47 g for an increment of 10 μg/m^3 in NO_2 exposure during pregnancy. We found significant mediated effects of prenatal exposure to NO_2 on birth weight through a decrease in mtDNA content (Proportion of mediation by mtDNA content: 9-12%).

Also, we identified mitochondrial DNA content as a susceptibility marker for the short-term effects of air pollution on HRV. Significant reductions in HRV with increasing PM concentrations were observed only in subjects with low mitochondrial DNA content, and no association with pollution was seen in subjects with high mitochondrial DNA content. Other studies identified that oxidative stress mediates the effects of air pollution on HRV. Drugs that modify oxidant defenses may influence the susceptibility to particle-induced inflammatory or pro-oxidative responses. In addition to their cholesterol lowering effect, statins also have potent anti-inflammatory properties. The

Normative Ageing study investigators found that the effect of PM_{10} on HRV was confined to persons missing the allele for GSTM1 (lower oxidative stress defense), but the association was only apparent in those who were not under statin treatment.

Further effect modification of anti-oxidative properties on association between air pollution and HRV has been shown by dietary antioxidants and B vitamins, as well as u-3 polyunsaturated fatty acids.[37-39] These findings imply that pathways that decrease endogenous oxidative stress have a protective effect that alleviates reductions in HRV due to exposure of particulate air pollution.

CONCLUSION AND PERSPECTIVES

PM exposure contributes to cardiovascular morbidity and mortality, particularly in the acute exposure period, as emphasized by the recent AHA statement on air pollution. The effect on shortening life expectancy has been estimated at 1-2 years for realistic exposure contrasts. In this doctoral dissertation we showed an overall diminishing effect of long-term PM air pollution on mitochondrial function in the context of the exposome and we identified mitochondrial function as a susceptibility marker for short-term exposure to PM.

Since mitochondrial DNA content is not a fixed characteristic, but a modifiable factor, this provides unique opportunities for modulation of the effect of air pollution. This feature is critical for pathologies related to non-preventable exposure, since air pollution is an ubiquitous public hefalth effect.
Further research is necessary to determine genetic, dietary and behavioral factors that can restore mitochondrial function.

Further, we emphasize the importance of children as a susceptible subgroup for the adverse effects of PM. PM-induced health effects are not limited to persons with underlying disease or elderly but affect the individual from conception onwards. Complications in adults often find their origin in risk factors operative early in life. However, we must admit that further research is necessary to determine the clinical consequences of short-term changes in cardiovascular parameters such as blood pressure and HRV in response to acute elevation in PM concentrations.

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CONFERENCE PRESENTATIONS AND PUBLISHED ABSTRACTS

Pieters N, Koppen G, Smeets K, Cuyypers A, De Prins S, Van De Weghe H, Nelen V, Cox B, Vangronsveld J, Schoeters G, Nawrot TS. Decreased mitochondrial DNA content in association with polycyclic aromatic hydrocarbons in winter time indoor house dust. Environmental Health across Land, Air and Sea, ISEE, Columbia, USA, 26-30 august 2012 **(oral presentation)**

Pieters N, Plusquin M, Cox B, Kicinski M, Vangronsveld J, Nawrot TS. An epidemiological appraisal of the association between heart rate variability and particulate air pollution: a meta-analysis. Environmental Health across Land, Air and Sea, ISEE, Columbia, USA, 26-30 august 2012 **(oral presentation)**

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